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**APPLICATION NUMBER: 60/532,667**

**FILING DATE: *December 24, 2003***

**RELATED PCT APPLICATION NUMBER: *PCT/US04/43087***



Certified By

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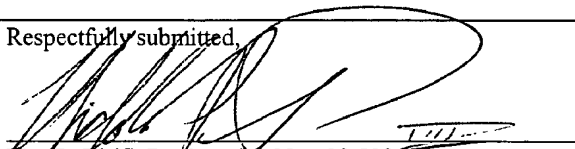
Attorney Docket No. 26069-530 PRO

**PROVISIONAL APPLICATION FOR PATENT COVER SHEET**

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR § 1.53(c)

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TITLE OF THE INVENTION			
SMMR (SMALL MOLECULE METABOLITE REPORTERS) FOR USE AS <i>IN VIVO</i> GLUCOSE BIOSENSORS			
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ENCLOSED APPLICATION PARTS			
<input checked="" type="checkbox"/> Specification		Number of Pages: 107	
<input type="checkbox"/> Sequence Listing		Number of Pages:	
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<input checked="" type="checkbox"/> No.			
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METHOD OF PAYMENT			
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<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge \$160.00 to cover the filing fees of the Provisional application to Deposit Account No. 50-0311, Reference No. 26069-530 PRO.			
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Respectfully submitted,



Date: December 24, 2003

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SEND TO: MAIL STOP PROVISIONAL APPLICATION, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-

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22388 U.S. PTO  
60/532667

The PTO did not receive the following  
listed item(s) 24 drawings, but  
received 19 drawings.

## **SMMR (SMALL MOLECULE METABOLITE REPORTERS) FOR USE AS *IN VIVO* GLUCOSE BIOSENSORS**

### **RELATED APPLICATIONS**

This application is related to pending U.S. Application No. 10/617,915, filed on July  
5 10, 2003 as Docket No. 26069-526; the entire contents of which are incorporated herein by  
reference.

### **FIELD OF THE INVENTION**

This invention provides compositions and methods for designing small molecule  
metabolite reporters (SMMRs) for optical reporting of cell metabolism and intracellular or  
10 extracellular metabolite or analyte concentrations. The specific application of this work is to  
design molecules that are able to optically report the concentration of the biologically active  
molecule D-glucose, including other small molecule analytes or metabolic processes, in or near  
human keratinocytes located within the viable epidermis of human or mammalian skin. The  
skin glucose levels are then used to infer blood glucose levels. In particular, this invention  
15 provides compositions and methods for several non-invasive techniques to determine *in vivo*  
blood glucose levels based upon the direct measurement of glucose levels present in the skin.

### **BACKGROUND OF THE INVENTION**

Identifying and understanding the risk factors associated with diabetes is invaluable for  
the development and evaluation of effective intervention strategies. Lacking normal regulatory  
20 mechanisms, diabetics are encouraged to strive for optimal control through a modulated life  
style approach that focuses on dietary control, exercise, and glucose self-testing with the timely  
administration of insulin or oral hypoglycemic medications. Invasive forms of self-testing are  
painful and fraught with a multitude of psychosocial hurdles, and are resisted by most  
diabetics. Alternatives to the currently available invasive blood glucose testing are highly  
25 desirable.

Conventional approaches seek to reduce or eliminate the skin trauma, pain, and blood  
waste associated with traditional invasive glucose monitoring technologies. In general, non-  
invasive optical blood glucose monitoring requires no samples and involves external irradiation  
with electromagnetic radiation and measurement of the resulting optical flux. Glucose levels  
30 are derived from the spectral information following comparison to reference spectra for glucose  
and background interferants, reference calibrants, and/or application of advanced signal

processing mathematical algorithms. Candidate radiation-based technologies include: 1) mid-infrared (MIR) spectroscopy, 2) near-infrared (NIR) spectroscopy, 3) far-infrared (FIR) spectroscopy, 4) radio wave impedance, 5) infrared photoacoustic spectroscopy and 6) Raman spectroscopy. Each of these methods uses optical sensors, and relies on the premise that the absorption pattern of infrared light (700-3000 nm) can be quantitatively related to the glucose concentration. Other substances such as water, protein, and hemoglobin are known to absorb infrared light at these wavelengths and easily obscure the relatively weak glucose signal.

Other approaches are based on microvascular changes in the retina; acoustical impedance, NMR spectroscopy and optical hydrogels that quantify glucose levels in tear fluid. While putatively non-invasive, these technologies have yet to be demonstrated as viable in clinical testing.

Nearly non-invasive techniques tend to rely on interstitial fluid extraction from skin. This can be accomplished using permeability enhancers, sweat inducers, and/or suction devices with or without the application of electrical current. One device recently approved by the FDA relies on reverse iontophoresis, utilizing an electrical current applied to the skin. The current pulls out salt, which carries water, which in turn carries glucose. The glucose concentration of this extracted fluid is measured and is proportional to that of blood. This technology, in keeping with its nearly non-invasive description, is commonly associated with some discomfort and requires at least twice daily calibrations against conventional blood glucose measurements (e.g., invasive lancing).

Other nearly non-invasive blood glucose monitoring techniques similarly involve transcutaneous harvesting for interstitial fluid measurement. Other technologies for disrupting the skin barrier to obtain interstitial fluid include: 1) dissolution with chemicals; 2) microporation with a laser, sound, or electrical stimulation; 3) penetration with a thin needle; and/or 4) suction with a pump. Minimally invasive blood glucose monitoring can also involve the insertion of an indwelling glucose monitor under the skin to measure the interstitial fluid glucose concentration. These monitors typically rely on optical or enzymatic sensors. Technologically innovative, these *in situ* sensors have had limited success. Implantable glucose oxidase sensors have been limited by local factors causing unstable signal output, whereas optical sensors must overcome signal obfuscation by blood constituents as well as interference by substances with absorption spectra similar to glucose. Moreover, inflammation associated with subcutaneous monitoring may contribute to systematic errors requiring

repositioning, recalibration or replacement, and more research is needed to evaluate the effects of variable local inflammation at the sensor implantation site on glucose concentration and transit time.

Interstitial fluid glucose concentrations have previously been shown to be similar to simultaneously measured fixed or fluctuating blood glucose concentrations (Bantle *et al.*, *Journal of Laboratory and Clinical Medicine* 130:436-441, 1997; Sternberg *et al.*, *Diabetes Care* 18:1266-1269, 1995). Such studies helped validate non-invasive/minimally invasive technologies for blood glucose monitoring, insofar as many of these technologies measure glucose in blood as well as interstitial fluid.

A non-invasive glucose monitor that is portable, simple and rapid to use, and that provides accurate clinical information is highly desirable. In particular, the ability to derive primary and secondary order information regarding real time, dynamic glucose metabolism (such as the direction and rate of change of bioavailable glucose distributed within the blood and interstitial fluid space) is highly desirable.

#### SUMMARY OF THE INVENTION

*In vivo* fluorescence (autofluorescence) has been used for a number of years to determine the metabolic state and to monitor pharmaceutical effects in cells and tissues (Dellinger *et al.*, *Biotechnol Appl Biochem*, 28(Pt. 1): 25-32, 1998). Consideration of the photophysics involved in autofluorescence rapidly leads one to the conclusion that the use of autofluorescence alone as the analytic probe imposes some severe limitations on any measurement technique.

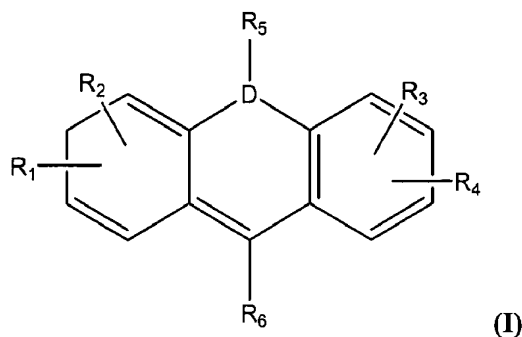
Fluorescence techniques are capable of detecting molecular species at picomole levels or less. This sensitivity arises because of the simplicity of detecting single photons against a dark background. This advantage disappears if there are other fluorescent species in the detection volume. Fluorescence intensity is also not an absolute technique and must be referenced to some internal standard using a ratiometric or comparative method.

It has been shown that fluorophores, or colored dyes utilizing absorption spectroscopy can be used to measure glucose in solution or serum by using series of separate reagents. These generic reagents include glucose oxidase (which oxidizes glucose forming hydrogen peroxide); peroxidase (generally, horseradish peroxidase: HRP) used to create an oxidizing reaction in the presence of hydrogen peroxide with the dye or fluorophore; and a dye reagent or

fluorophore, which changes its color or fluorescence spectrum when brought in contact with hydrogen peroxide, and peroxidase. The resultant colored or fluorescent species is measured with a colorimeter or fluorimeter and the amount of glucose in solution is calculated. In addition, other analytical techniques have been shown to be commercially useful for measuring hydrogen peroxide generated from the reaction of glucose oxidase and glucose.

The methods and compositions of the present invention effectively determine the glucose concentration in blood for a living organism by non-invasive, *in vivo* measurement of the glucose level in skin by means of fluorescence measurements of metabolic indicators/reporters of glucose metabolism. Disclosed are dyes used as metabolic indicators that allow for specific *in vivo* monitoring of metabolites, which are used as indicators of metabolic activity. Dyes characterized by this invention are referred to herein as a small molecule metabolite reporters ("SMMRs").

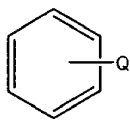
The invention relates in one aspect to SMMRs which comprise novel xanthene-based boronic acid compounds. The SMMRs may be used in sensor compositions for, *e.g.*, direct measurement of glucose, and in other diagnostic or analytical methods as described herein. In an embodiment these compounds are of the following formula (I):



wherein

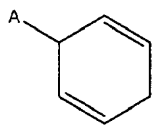
- D is a heteroatom;
- R<sub>1</sub> and R<sub>2</sub> are different and are selected from the group consisting of H, OH, NH<sub>2</sub>, NO<sub>2</sub>, OCH<sub>3</sub>, N(CH<sub>3</sub>)<sub>2</sub>, A, or R<sub>1</sub> and R<sub>2</sub>, taken together with the ring to which they are attached, form R<sub>7</sub>;
- R<sub>3</sub> and R<sub>4</sub> are different and are selected from the group consisting of H,  $\text{C}(=\text{O})\text{OH}$ , B(OH)<sub>2</sub>, M, or R<sub>3</sub> and R<sub>4</sub>, taken together with the ring to which they are attached, form R<sub>8</sub>;

- $R_5$  and  $R_6$  are different and are selected from the group consisting of H or

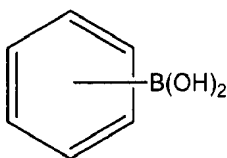


, wherein Q is H, COOH, B(OH)<sub>2</sub>, or M;

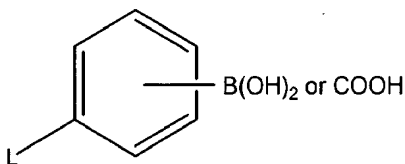
- A is OH, NH<sub>3</sub>, or ;



- $R_7$  is



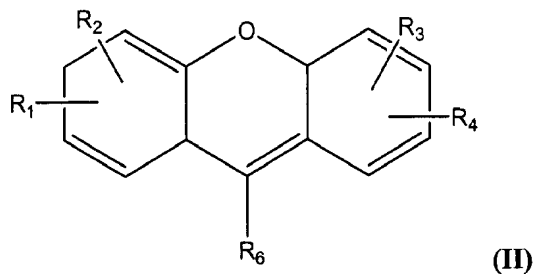
- $R_8$  is



- M is ;
- L, when present, is an amino-containing linking moiety;
- $R_1$  and  $R_2$ , and  $R_3$  and  $R_4$ , are adjacent to each other on the rings on which they reside; and
- at least one boronic acid moiety is present; and salts thereof.

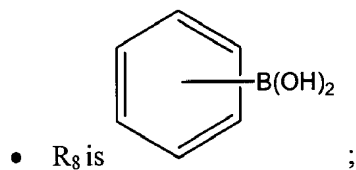
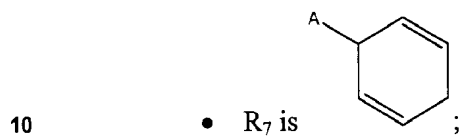
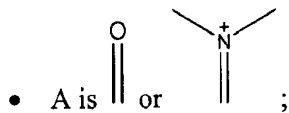
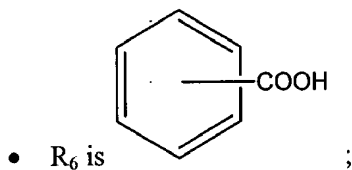
“Amino-containing linking moieties” may include moieties comprising a substituted or unsubstituted amino group, an amido group or a sulfonamido group. In an embodiment, xanthene-based boronic acid compounds of the invention include those of the formula (II):

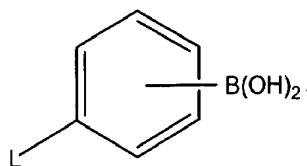




wherein

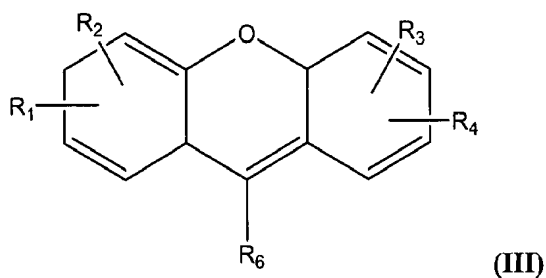
- $R_1$  and  $R_2$  are different and may be A, or,  $R_1$  and  $R_2$ , taken together with the ring to which they are attached, form  $R_7$ ;
- 5      •  $R_3$  and  $R_4$  are different and are selected from the group consisting of  $H$ ,  $\overset{O}{\parallel}$ ,  $OH$ ,  $B(OH)_2$ , M, or  $R_3$  and  $R_4$ , taken together with the ring to which they are attached, form  $R_8$ ;






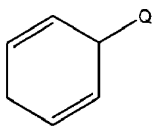
- M is ;
- L, when present, is an amino-containing linking moiety; and
- R<sub>1</sub> and R<sub>2</sub>, and R<sub>3</sub> and R<sub>4</sub>, are adjacent to each other on the rings on which they reside; and salts thereof.

5 In another embodiment, xanthene-based boronic acid compounds of the invention include those of the formula (III):


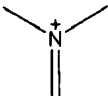


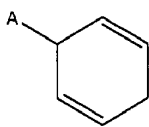
wherein

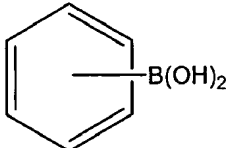
- R<sub>1</sub> and R<sub>2</sub> are different and are selected from the group consisting of H, OH, NH<sub>2</sub>, NO<sub>2</sub>, OCH<sub>3</sub>, N(CH<sub>3</sub>)<sub>2</sub>, A, or, R<sub>1</sub> and R<sub>2</sub>, taken together with the ring to which they are attached, form R<sub>7</sub>;
- R<sub>3</sub> and R<sub>4</sub> are different and are selected from the group consisting of H, , OH, M, or R<sub>3</sub> and R<sub>4</sub>, taken together with the ring to which they are attached, form R<sub>8</sub>;
- R<sub>5</sub> and R<sub>6</sub> are different and are selected from the group consisting of H or

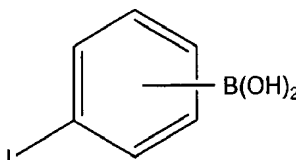


, wherein Q is H or M;

- A is OH, NH<sub>3</sub>,  or  ;

- R<sub>7</sub> is  ;

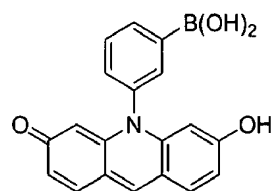
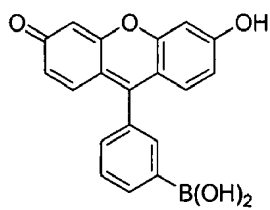
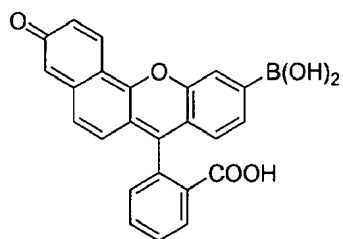
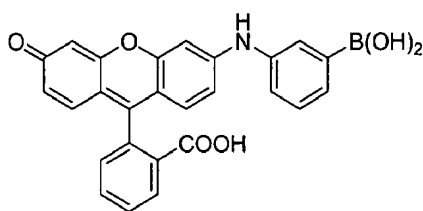
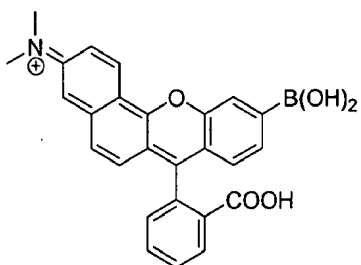
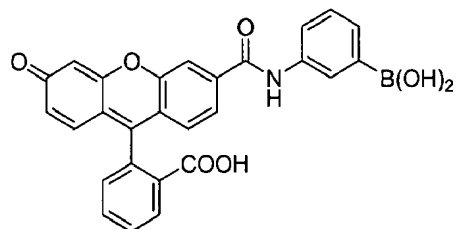
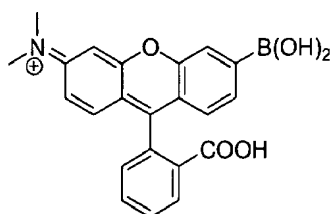
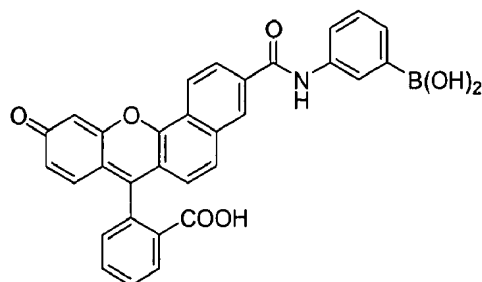
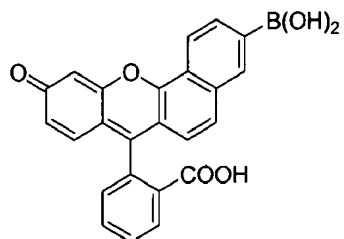
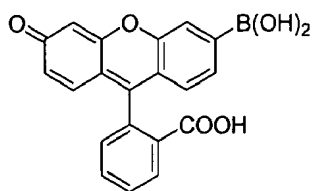
- R<sub>8</sub> is  ;

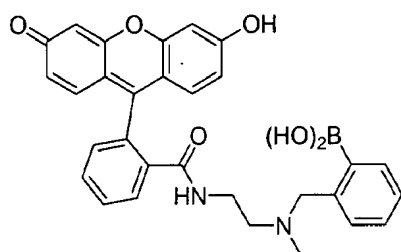
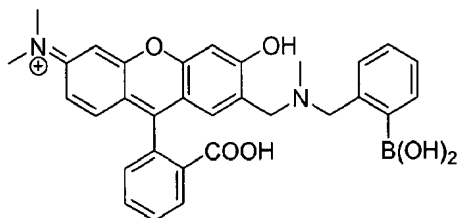
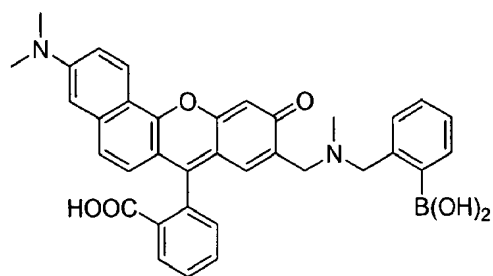
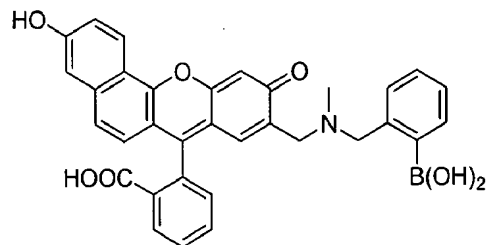
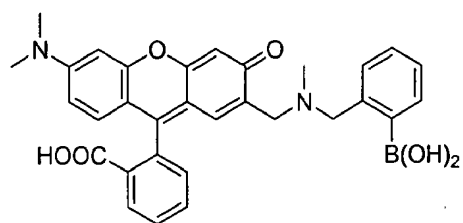
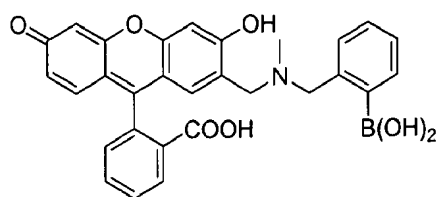
- M is  ;

5

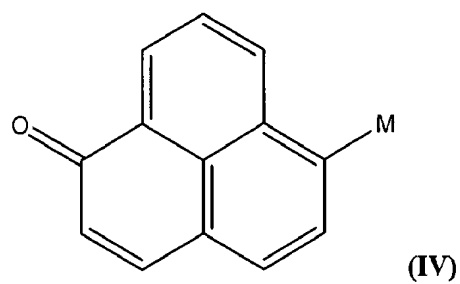
- L, when present, is an amino-containing linking moiety; and
- R<sub>1</sub> and R<sub>2</sub>, and R<sub>3</sub> and R<sub>4</sub>, are adjacent to each other on the rings on which they reside; and salts thereof.

Examples of xanthene-based SMMRs of the invention include:

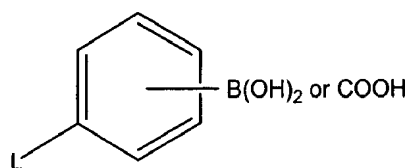




The invention relates in another aspect to SMMRs which comprise novel phenalene-based boronic acid compounds. In an embodiment these compounds are phenalene-1-one compounds of the following formula (IV):



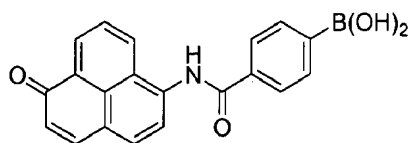
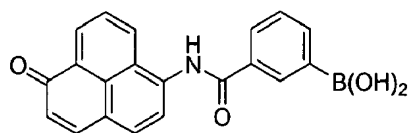
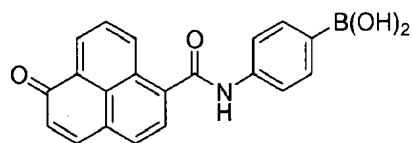
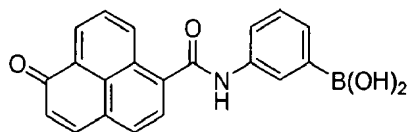
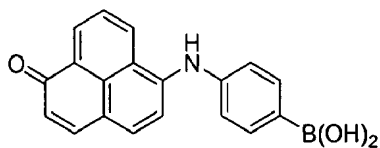
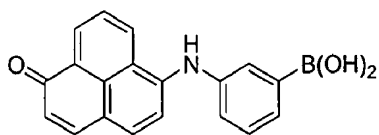
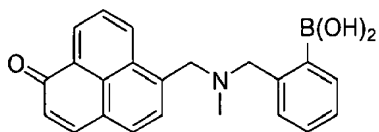
wherein



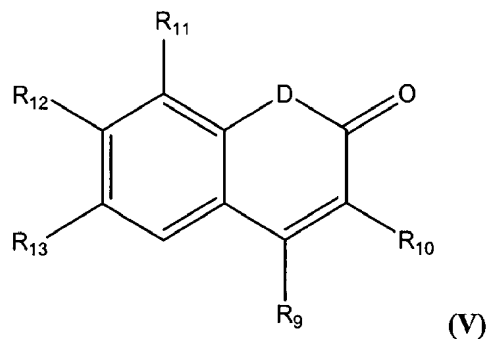
- M is ; and
- L, when present, is an amino-containing linking moiety; and salts thereof.

5

Examples of such phenalene-based boronic acid compounds include:



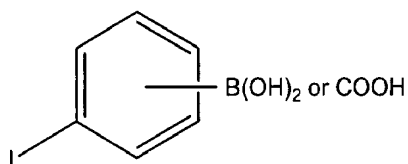
The invention relates in another aspect to SMMRs which comprise novel boronic acid-containing coumarin or carbostyryl derivative compounds. In an embodiment these compounds include those of the following formula (V):



5 wherein

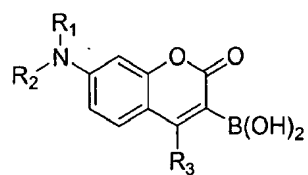
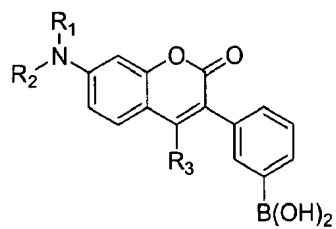
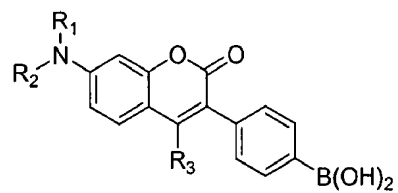
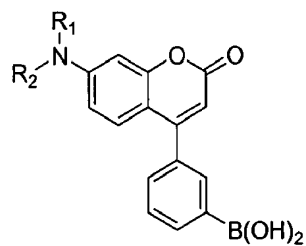
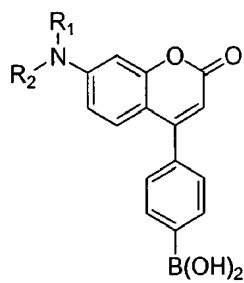
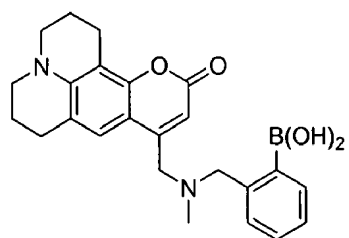
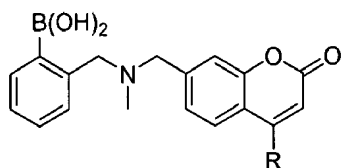
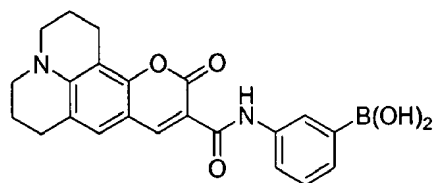
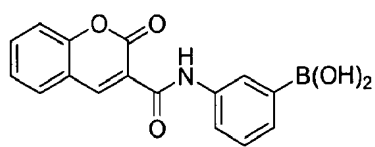
- D is a heteroatom (*e.g.*, O or N);
- R<sub>9</sub> is H, OH, CH<sub>3</sub>, CF<sub>3</sub>, M, or an amino or substituted amino group;
- R<sub>10</sub> is H, CH<sub>3</sub>, or M;
- R<sub>11</sub>, R<sub>12</sub>, and R<sub>13</sub> are individually H, OH, alkoxy, M, or an amino or substituted amino group;
- R<sub>14</sub>, when present, is H or CH<sub>3</sub>;

10

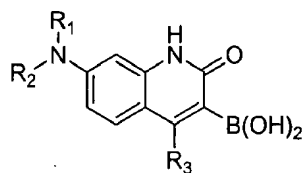
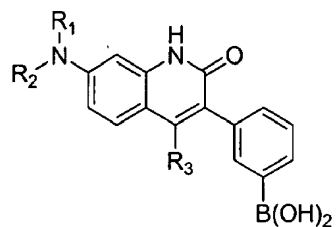
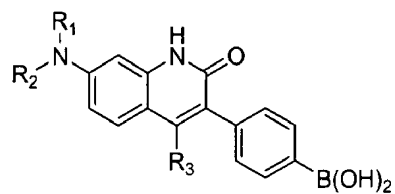
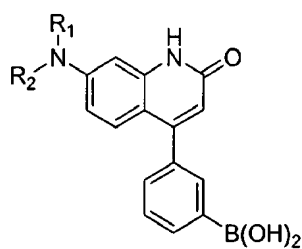
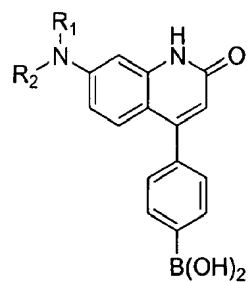
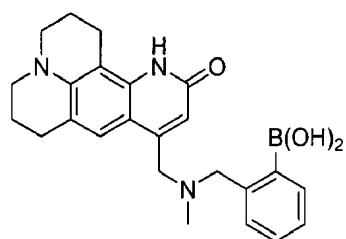
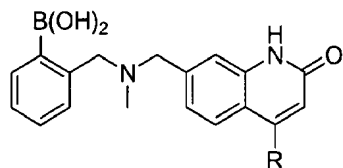
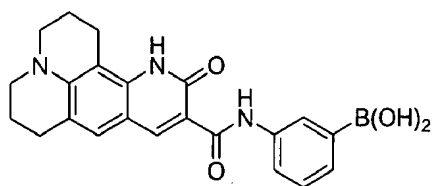
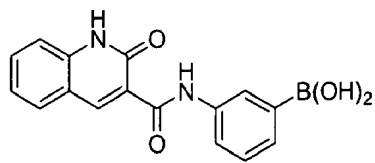


- M is ; and
- at least one boronic acid moiety is present; and salts thereof.

15 A substituted amino group may include where R<sub>11</sub>, R<sub>12</sub>, and R<sub>13</sub>, taken together with the ring to which they are attached, form a nitrogen-containing polycycle. Examples of such boronic acid-containing coumarin or carbostyryl SMMRs include:



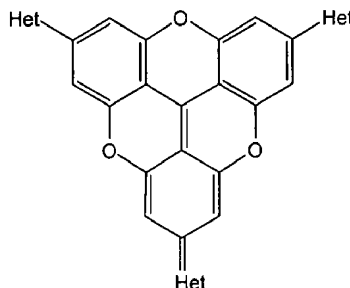




It will be noted that the structure of some of the compounds of the invention includes asymmetric carbon atoms. It is to be understood accordingly that the isomers arising from such asymmetry (*e.g.*, all enantiomers and diastereomers) are included within the scope of the

invention, unless indicated otherwise. Such isomers can be obtained in substantially pure form by classical separation techniques and by stereochemically controlled synthesis. Alkenes can include either the E- or Z-geometry, where appropriate. Tautomeric forms of compounds of the invention are also intended to be included within the scope of the invention, unless  
 5 indicated otherwise.

In another aspect, a chromophore of the following rational design structure is disclosed:



wherein Het represents a heteroatomic group, *e.g.*, containing N, O, or S; B(OH)<sub>2</sub>; M or R<sub>8</sub> (as set forth in Formula (I)); or mono or di-substituted N, NO<sub>2</sub> or N(CH<sub>3</sub>)<sub>2</sub>; which groups  
 10 may be identical or different. Heteroatomic groups may include amino, amido, carbonyl, hydroxyl, thiol, and thio.

This invention also provides for fluorescence measurements of extracellular and intracellular reporter molecules placed into the cytosol, nucleus, or organelles of cells within intact, living, tissue that track the concentration of blood glucose in an organism. When any  
 15 one of a series of metabolites is measured using this technique, the molar concentration of blood glucose can be calculated. Direct or indirect fluorescence measurements of glucose using one or more of the following measurements is described: pH (as lactate/H<sup>+</sup>), membrane reduction-oxidation electric potential, NAD(P)H (nicotinamide adenine dinucleotide (phosphate), reduced form) for energy transfer, FAD<sup>+</sup> (flavin adenine dinucleotide, oxidized  
 20 form) for energy transfer, ATP/ADP ratio, Ca<sup>2+</sup>-pumping rate, Mg<sup>2+</sup>-pumping rate, Na<sup>+</sup>-pumping rate, K<sup>+</sup>-pumping rate, and vital mitochondrial membrane stains/dyes/molecules fluorescence response. These analytes, measured in skin using the techniques taught herein, are used to provide a complete picture of epidermal skin glycolytic metabolism where local epidermal analyte (glucose) quantities are proportional to the concentration of glucose in  
 25 systemic blood, specifically the capillary fields within the papillary layer of the dermis (corium). Temperature and/or nitric oxide measurement may also be combined with the above measurements for better calibration and determination of glucose concentrations.

The invention further provides sensor compositions that are applied to at least one surface of living tissue, organs, interstitial fluid, and whole organisms and transported into the tissue at an effective concentration. The sensor composition can include at least one SMMR at an effective concentration such that when the at least one SMMR is brought in contact with one or more specific metabolites or analytes, a change in fluorescence or absorption occurs, thereby allowing quantification of the change in fluorescence or absorption.

The primary embodiment of this invention utilizes a series of molecules (SMMRs) specifically designed for topical delivery onto tissue, such as the viable epidermis, which when applied to the tissue will report glucose concentration using any one or more of several reporting mechanisms. The most significant advantage of the present invention is increased sensitivity in reporting glucose concentration, while eliminating the requirement to draw body fluid from the skin as is required by current conventional techniques.

The *in vivo* information obtained when the SMMR is brought in contact with the one or more metabolites or analytes can include, but is not limited to, assessment of metabolic function; diagnosis of metabolic disease state; monitoring and control of disease state; stress status of cells, tissues and organs; determination of vitality and viability of cells based on metabolic function; critical care monitoring; diagnosis and monitoring of cardiovascular diseases, autoimmune disorders, neurological disorders, degenerative diseases; determination of metabolic concentration; and cancer diagnosis, detection, staging and prognosis.

For example, the *in vivo* information obtained may provide detailed information on glucose metabolism, fructose metabolism and galactose metabolism; advanced-glycosylated end products; monitoring and control of diseases such as diabetes, cancer, stress and organ transplantation.

The sensor compositions used in these methods for monitoring the concentration of one or more metabolite(s) or analyte(s) can be formulated as, but are not limited to, emulsions, ointments, disposable gel film patches, reservoir devices, creams, paints, polar solvents, non-polar solvents, or any combination thereof.

Penetration of the sensor composition can be accomplished using an active transport technique or a passive transport technique, such as, for example, electroporation, laser poration, sonic poration, ultrasonic poration, iontophoresis, mechanical-poration, solvent transport, tattooing, wicking, microneedle or pressurized delivery. In addition, penetration of the sensor

composition to the desired depth can be accomplished by combining the composition with various molecular size attachments.

Typically, the quantification of the change in fluorescence or absorption is monitored using fluorescence or absorption spectroscopy.

5           An effective concentration of the sensor composition is, for example, at least between 0.01 to 500  $\mu\text{g/ml}$ , between 0.1 to 500  $\mu\text{g/ml}$ , between 1.0 to 150  $\mu\text{g/ml}$ , between 1 to 100  $\mu\text{g/ml}$ , and between 10 to 100  $\mu\text{g/ml}$ . The SMMR can be introduced in a low concentration in a range from 10  $\mu\text{M}$  to 1000  $\mu\text{M}$  and in a volume from 200  $\mu\text{L}$  to 0.1  $\mu\text{L}$ , respectively (e.g., introducing the SMMR at a concentration in the range of 200 $\mu\text{L}$  of a 10  $\mu\text{M}$  SMMR solution to  
10   0.1  $\mu\text{l}$  of a 1000  $\mu\text{M}$  SMMR solution). One specific application of the sensor composition is, for example, a 5  $\mu\text{L}$  volume of a 400  $\mu\text{M}$  SMMR solution, or a 10  $\mu\text{L}$  volume at 200  $\mu\text{M}$  concentration.

Once one or more SMMRs are activated as a result of placement within the skin, fluorescence measurements monitor the response of the skin cells to glucose. As described  
15   herein, the fluorescence mechanism used is either a direct or indirect indication of the glucose concentration in the target cell environment. Fluorescence is typically measured using an optical reader. The optical reader calculates the skin response to glucose, applies first principles mathematical models to the response, and provides a determination of the blood glucose levels. Choosing the particular commercially available or custom designed optical  
20   reader that is compatible for use with the methods and compositions of this invention is within the ability of one skilled in the art.

One embodiment of this invention utilizes indirect means to measure skin and blood glucose *in vivo* by placing one or more SMMRs into the viable epidermis to form a fluorescent product. This fluorescent product is provided by one of many specifically described reporting  
25   mechanisms, whereby the SMMR fluorescent signal changes with respect to the effects of glucose concentration on cell metabolism. The quantity of fluorescence, or the fluorescent ratio at two or more emission wavelengths, is indicative of the total glucose concentration within the skin, either intracellular or extracellular as described here. The skin glucose thus determined is used to infer blood glucose levels as calibrated and described herein.

30           In another embodiment, a method for monitoring *in vivo* blood glucose levels uses SMMRs that directly bind or respond to glucose itself. The mechanisms of glucose reporting

thus does not use cell metabolism, as in the first embodiment, but rather the SMMR responds to glucose by one of several direct mechanisms to produce a fluorescent product. The measured fluorescence is thus a direct reporter of the interstitial fluid or extracellular glucose concentrations. Thus, the skin glucose level directly determined *in vivo* is used to infer blood glucose levels as calibrated and described within this invention text.

This invention describes the unique physicochemical, photochemical, photophysical and biological properties of SMMR molecules, as well as their design, synthesis, and application. The use of an SMMR enables fluorescence measurements from picomolar through millimolar *in vivo* glucose levels in living skin tissue, or interstitial fluid, either of which are indicative of the blood glucose levels. The invention described here relates to the indirect or direct determination of skin glucose levels for use in the monitoring and control of diabetes mellitus. Embodiments of the invention use SMMR fluorescence to measure skin glucose levels without withdrawing bodily fluids. When the SMMR-based skin glucose measurements are made, the blood glucose levels are directly inferred.

The quantity of glucose in the epidermis is supplied by mass transport from the blood vessels and capillary fields located within the dermis, immediately beneath the epidermis. The movement of glucose from the blood stream to the epidermis is concentration dependent and non-insulin regulated providing the basis for measurement of blood glucose as a direct inference from skin glucose measurement. The rate of glucose transport into the epidermis is indicative of the differential concentration between skin glucose and blood glucose levels. The rate of transport into the extracellular spaces between human skin cells allows an accurate first principles mathematical extrapolation of blood glucose levels. Once modeled, the kinetics of blood glucose transport to the skin from the blood enables the determination of the precise first principles mathematical relationship between the rate of change of skin glucose and the rate of change of blood glucose. Thus rapid blood glucose concentration changes up or down can be accurately tracked by knowing the skin glucose mean concentration levels and the rate of change of skin glucose levels. First principles mathematical models can be developed for the individual case, preferably for small local populations, and most preferably for a universal patient case.

The SMMR-derived fluorescence reports glucose levels within or surrounding human keratinocyte cells as an indication of blood glucose levels. The movement of glucose from the interstitial fluid surrounding the keratinocytes into the keratinocytes of the epidermis is

concentration dependent and non-insulin regulated. That is, the glucose is transported into these cells via non-insulin regulated glucose transporter GluT1 (GenBank Accession Number: K03195), not insulin regulated glucose transporter GluT4 (GenBank Accession Number: M91463). This transport mechanism provides the basis for measurement of blood glucose as a  
5 direct inference from intracellular keratinocyte glucose measurement.

Also provided are noninvasive methods for monitoring *in vivo* blood glucose levels. According to these methods at least one small molecule metabolic reporter is applied to at least one surface of skin for a predetermined period of time causing penetration of the one or more SMMRs to a depth of about 10  $\mu\text{m}$ , wherein the depth corresponds with the bottom of the dead  
10 stratum corneum layer, to about 175  $\mu\text{m}$ , wherein the depth corresponds with the top of the dermal layer, into the epidermis. The one or more SMMRs come in contact with one or more metabolites or analytes and a change in the concentration of the one or more metabolites or analytes is monitored by detecting changes in the SMMRs using an optical reader. The change in the concentration of the one or more metabolites or analytes is then correlated with *in vivo*  
15 blood glucose levels.

Also included in the invention is a reagent strip for use in a glucose measuring instrument comprising a polymer strip and a known concentration of at least one small molecule metabolic reporter, wherein when a sample of a biological fluid containing an amount of glucose is interacted with the reagent strip, a change in fluorescence or absorption of the one  
20 or more molecular sensor proteins occurs, and the change is measured by the glucose measuring instrument, thereby detecting the glucose concentration of the biological fluid.

The change in fluorescence or absorption can be monitored using fluorescence or absorption spectroscopy. Those of ordinary skill in the art will recognize that any fluorescence or absorption spectroscopic techniques can be used in accordance with the invention.

25 The invention also provides sensor systems that include a device having a component that transmits radiation to a material or tissue, a component that detects radiation emitted from a material or tissue, and a component to display the detection results, each component is operably linked. The sensor systems further include an applicator that delivers the sensor composition of the invention to the material or tissue. Typically, there is an air interface  
30 between the device and the material or tissue, wherein the air interface measures a resulting excitation radiation emitted from the irradiated sensor composition.

The device included in the sensor system can emit radiation at one or more wavelengths that have been chosen to specifically excite the SMMR mixture that is applied to the material or tissue. The sensor composition can include a reporter dye and a marker dye, or alternatively, a dye exhibiting a wavelength shift in absorption or fluorescence emission in the presence of a metabolite. The sensor composition can be present at a depth from the surface of the skin of about 10  $\mu\text{m}$  to about 175  $\mu\text{m}$  in the epidermis in a concentration that is effective for detection of one or more metabolites or analytes in a biological sample.

The sensor system can detect radiation at one or more wavelengths that have been chosen to specifically identify fluorescence emission that has been scattered back to the system from the sensor composition.

### BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Glucose pathway of living cells

FIG. 2. Summary of photochemical reaction pathways

FIG. 3. Absorption and emission spectra for 5  $\mu\text{M}$  of an example ratiometric pH reporting dye in PBS. Fluorescence spectra were measured using 532 nm to excite the dye.

FIG. 4. Fluorescence emission spectra of an example ratiometric pH reporting dye loaded into the A-431 cells. Nigericin is present and allows intracellular pH to equilibrate extracellular pH.

FIG. 5. "Absolute" and normalized Fluorescence, auto-Fluorescence and Air spectra.

FIG. 6. Difference and relative spectra of normalized Fluorescence spectra.

FIG. 7. Glucose, Lactate and SMMR measured ratio kinetics.

FIG. 8. Kinetics of physiological parameters and ratio.

FIG. 9. Normalized and relative changes of ratio, glucose, and lactate in time.

FIG. 10. Fluorescence ratio changes after anesthesia (0.1 cc of ketamine).

FIG. 11a. Data from a clamp study, comparison between experimental (YSI values) and calculated values of  $\Delta R$  (from the equation  $\Delta R = \alpha \Delta G + \beta \Delta L$  using the coefficients  $\alpha$  and  $\beta$  from above).

FIG. 11b. Data from another clamp study, comparison between experimental (YSI values) and calculated values of  $\Delta R$  (from the equation  $\Delta R = \alpha\Delta G + \beta\Delta L + \Delta R_a(t-t_a)$  A using the coefficients  $\alpha$ ,  $\beta$  and  $\Delta R_a(t-t_a)$  from above.

FIG. 11c. Data from yet another clamp study, comparison between experimental (YSI values) and calculated values of  $\Delta R$  (from the  $\Delta R = \alpha\Delta G + \beta\Delta L + \Delta R_a(t-t_a)$  A using the coefficients  $\alpha$ ,  $\beta$  and  $\Delta R_a(t-t_a)$  from above.

FIG. 12. NADH peak fluorescence signals versus time as glucose concentration is changed. Glucose concentrations are annotated in the Figure. The first annotation with 50% PBS is a control measurement.

FIG. 13. Glucose concentration is varied from 0 to 66 mg/dL and the NADH peak signals exhibit corresponding changes in intensity.

FIG. 14. NADH measurements versus sequence number. The glucose values (in mg/dL) for each data point are (from left to right): 0, 0.1, 0.5, 1.0, 2.0, 5.0, 2.5, 1.0, 0.5, 0.25, 0.16, and 4.0 mg/dL. Each point represents the mean and standard deviation of 5 data points.

NOTE: The x-axis label sequence number.

FIG. 15. Corrected NADH Signal of Figure14 for each data point (as from left to right), corresponding to a concentration 0, 0.1, 0.5, 1.0, 2.0, 5.0, 2.5, 1.0, 0.5, 0.25, 0.16, and 4.0.

FIG. 16. Corrected NADH Signal versus Glucose concentration for upward trend and downward trend data together.

FIG. 17. A membrane-bound Rh123 peak fluorescence signal change (decrease in signal as an indication of membrane potential change) versus time as glucose concentration is changed. Glucose concentrations were increased at the 15-minute point in the Figure. The first annotation with 50% PBS is a control measurement.

FIG. 18. Glucose concentration is varied from 0 to 66 mg/dL and the Rh123 (bottom plot) peak signals exhibit corresponding changes in intensity due to membrane potential changes.

FIG. 19. Rh123 fluorescence quenching (as an indication of membrane potential changes) versus sequence number and glucose concentration. The glucose values (in mg/dL) for each data point is (from left to right): 0, 0.1, 0.5, 1.0, 2.0, 5.0, 2.5, 1.0, 0.5, 0.25, 0.16, and



4.0. Each point represents the mean and standard deviation of 5 data points. The x-axis label is sequence number.

FIG. 20. Schematic representation of the products of the xanthene library in terms of Markush structures and some specific sub structures.

5 FIG. 21a. Absorption and emission spectra for BeXan type dyes.

FIG. 21b. Absorption and emission spectra for fluorescein.

FIG. 22. Malachite green absorption spectrum (left), with molecular structure (right).

FIG. 23. Rhodamine B absorption spectrum (left), with molecular structure (right).

### DETAILED DESCRIPTION OF THE INVENTION

10 The features and other details of the invention will now be more particularly described with reference to the accompanying drawings and pointed out in the claims. It will be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention. All parts and  
15 percentages are by weight unless otherwise specified.

#### *Definitions*

The singular forms “a”, “and” and “the” include plural referents unless the context clearly dictates otherwise. For example, the term small molecule metabolic reporter “SMMR” includes one or more small molecule metabolic reporters “SMMRs”. Those skilled in the art  
20 will recognize that the terms “SMMR” and “SMMRs” are used interchangeably herein.

The term “biologically active molecule” includes, but is not limited to, enzymes, coenzymes, metabolites, analytes, reactive species, polypeptides, proteins, cofactors, small molecules and other macromolecules of physiological significance including mixtures or active fragments or subunits thereof. A “small molecule” includes a molecule from 100 Da to 250  
25 kDa. Molecules of this molecular weight range have a demonstrated ability for use as quantitative reporters of glucose activity.

The terms “small molecule metabolic reporter(s)”, “SMMR(s)”, “analyte enhancing molecules”, “reporter” and “reporters” include, but are not limited to, fluorophores, protein-labeled fluorophores, proteins with a photooxidizable cofactor (such as FADH  
30 contained in a glucose oxidase), and proteins with another intercalated fluorophore.

A “chromophore” includes a molecule exhibiting specific absorption or fluorescence emission when excited by energy from an external source. This is a more generic term than fluorophore.

5 A “fluorophore” includes a molecule exhibiting specific fluorescence emission when excited by energy from an external source.

An “intercalated fluorophore” includes fluorophores that will fluoresce when intercalated with a molecule. For example, Glucose Oxidase-Intercalated Fluorophore (GO-IF) is a molecule with specific glucose binding sites. The fluorescent properties will change when glucose binds to the molecule, causing a measurable change.

10 A “dye” includes molecules having large absorptivity or high fluorescence quantum yield and which demonstrates affinity for certain materials or organic (cellular) structures.

A “xanthene dye” includes a molecule having a xanthene-like skeletal structure, which exhibits large absorptivity and high fluorescence quantum yield and which demonstrates affinity for certain materials or organic (cellular) structures.

15 The phrase “energy transfer from reducing equivalents (*e.g.*, NAD/NADH, NAD(P)/NAD(P)H, FAD/FADH<sub>2</sub>) indicating SMMRs” refers to a use of SMMRs whereby the presence of these reducing equivalents molecules, is detected by excitation of the reducing equivalents molecules from an external source, energy transfer from the reducing equivalents molecule(s) to an SMMR, and detection of the fluorescence emission at the SMMR emission  
20 wavelength.

The phrase “absorption/diffuse reflection or fluorescence spectrum” refers to two types of spectra measured independently. The absorption/diffuse reflection spectrum refers to the energy reflection spectrum from a material reported in either the dimensions of reflectance or absorbance versus wavelength. The fluorescence spectrum is measured independently as the  
25 fluorescence emission intensity or the fluorescence lifetime of a fluorophore following excitation from an external source.

The phrase “molecular size attachment” refers to the molecular size in Angstroms (Å), which is related to molecular weight in Daltons (Da), of an attachment added as an adjunct to an SMMR. As used herein, “molecular size attachments” includes adducts to the fluorescent  
30 moieties of SMMRs that include, but are not limited to, structural modifications of fluorescence SMMRs as the additions to the fluorescence structure of: acetoxymethyl esters,

chloro-methyl derivatives, alkyl chain adducts, highly charged moieties, enzyme substrate mimics, enzyme cofactor tethers, and membrane binding tethers.

As used herein, a “reporter” includes an SMMR having the property of optical or fluorescence signal related to the quantity of analyte in the immediate vicinity of the SMMR.

5 Thus, as the analyte quantity increases, the fluorescence signal changes (up or down) in proportion.

As used herein, a “marker” includes a molecule having the property of yielding a fluorescence signal that is constant when applied to target cells or tissues. Its main purpose is for use as a reference signal channel. As such, it is applied in a ratiometric measurement for  
10 correction of a reporter signal. The variation in physiological and optical characteristics of individual subjects requires a reference channel signal to correct or normalize a reporter channel signal when the ratio of reporter to marker is used for quantitative applications.

As used herein, a “sensor” includes a handheld device capable of making absorption or fluorescence measurements at one or more wavelengths, and converting the ratios and sums of  
15 these measurements into analyte concentrations. These analyte concentrations are used to infer the rate or quantity of a specific metabolic process.

As used herein, a “metabolite” includes a substance produced by a metabolic process, such as glycolysis, which can be quantitatively measured as an indication of the rate or quantity of a specific metabolic process.

20 As used herein, an “analyte” includes a measurable parameter, using analytical chemistry, which can be quantitatively measured as an indication of the rate and quantity of a specific metabolic process. The term analyte is a generic term describing such concepts as metabolites, ions, processes, conditions, physico-chemical parameters, or metabolic results that can be used to infer the rate or quantity of specific metabolic processes.

25 As used herein, a “response range” includes an analyte range (lower and upper limits) over which a metabolic process, and its measured absorption or fluorescence signal, follow a linear or defined mathematical function.

The phrase “physico-chemical parameter” refers to a subset of broadly defined analyte parameters specifically related to the physical chemistry constants of materials. These  
30 constants can be used in combination with the measurement of other analytes to infer the rate

or quantity of specific metabolic processes. Such constants refer specifically to, *e.g.*, atomic mass, Faraday constant, Boltzmann constant, molar volume, dielectric properties, and the like.

As used herein, “wicking” includes the flow of a liquid into a solid material via the pull of gravity, Brownian motion, adhesion, mass transport, or capillary action such that a natural  
5 movement of a liquid occurs into a solid material.

The phrases “direct metabolic reporters,” and “indirect metabolic reporters” refer to the mechanism of action of SMMR for reporting glucose concentration. Direct metabolic reporters report the concentration of glucose directly, whereas indirect metabolic reporters report the concentration of analytes used to infer the concentration of glucose.

10 As used herein, an “octanol-water coefficient ( $K_{ow}$ )” includes a measure of the extent to which a solute molecule is distributed between water and octanol in a mixture. The octanol-water partition coefficient is the ratio of a chemical's solubility (concentration) in octanol to that in water using a two-phase mixture at equilibrium.

As used herein, “toxicity” includes the degree or quality of being toxic or hazardous to  
15 the health and well being of human and other mammalian organisms, organs, tissues, and cells.

The phrase “specialized tattoo” or more precisely the “active viewing window” refers to an area of tissue treated with an SMMR. That area is used for viewing the fluorescence ratio measurements of the SMMR interaction with tissue, in order to directly measure, calculate, or otherwise infer the concentration of skin and blood glucose or other metabolites of interest.

20 As used herein, “organ” includes a structure that contains at least two different types of tissue functioning together for a common purpose. Examples of organs in the body include, but are not limited to, the brain, heart, liver, kidneys, pancreas, stomach, intestines, lungs, and skin.

As used herein, a “keratinocyte” includes a living cell comprising the majority of the  
25 epidermis of mammalian skin. The keratinocyte is unique in both its proximity to the surface of an organism as well as in its glycolytic behavior. The keratinocyte metabolizes glucose in such a way as to produce a number of analytes whereby the glucose concentration within the cell can be inferred.

As used herein, a “mammal” includes both a human and a non-human mammal (*e.g.*, rabbit, mouse, rat, gerbil, cow, horse, sheep, etc.). Transgenic animals are also encompassed within the scope of the term.

The non-invasive devices, compositions, and methods of the present invention directly yield *in vivo* information for the assessment of intracellular and extracellular metabolic state, as well as the stress status of cells, tissues, and organisms. In a preferred embodiment, the devices, compositions and methods of the invention can be used to monitor and determine metabolite concentration levels, and more specifically, determine blood glucose concentration levels.

The invention provides non-invasive sensor compositions that comprise one or more small molecule metabolic reporters (“SMMRs” or “reporters”). When applied topically to skin, peripheral tissues, or organs, these reporters are able to penetrate the upper tissue layers and interact with a specific biologically active molecule in such a way as to report metabolic or health status, while not interfering with metabolic function. The reporters provide a metabolic signal that can be used for multiple purposes including, but not limited to, assessment of metabolic function (*e.g.*, particularly as related to glucose metabolism); diagnosis of metabolic disease states (*e.g.*, as related to advanced glycosylated end-products); monitoring and control of disease state; stress status of cells, tissues and organs; determination of vitality and viability of cells based on metabolic function; critical care monitoring; diagnosis and monitoring of cardiovascular diseases, autoimmune disorders, neurological disorders, degenerative diseases; determination of metabolic concentration; and cancer diagnosis, detection, staging and prognosis. Specifically, applying the reporters of the invention to living peripheral or epithelial tissue provides detailed information on the state of multiple metabolic pathways in living organisms that can be analyzed using low-cost, hand held instrumentation.

The invention provides techniques whereby one or more reporters are applied to solid tissue (*i.e.*, are introduced to the upper cell layers of tissues and organisms following local and/or topical administration). The reporters are added in trace quantities (from about 10 to about 1000  $\mu$ L of 0.1 to 200  $\mu$ M, preferably from about 5 to about 100  $\mu$ L), using a substance that is transparent to visible light and that has a pre-specified temporary residence at the application site (*e.g.*, 2 days-up to 30 days, 24-48 hours, preferably 2-6 hours, more preferably 30 seconds to 5 minutes, and most preferably 5 seconds to 5 minutes). Contemplated diffusion times include periods less than 48 hrs, 24 hrs, 10 hrs, 6 hrs, 2 hrs, 1 hr, 30 min, 15 min, 10 min,

5 min, 1 min, 30 sec, 10 sec, or 1 sec. Reporters that are placed on skin are able to penetrate the skin and be transported to a depth from the surface of from about 10  $\mu\text{m}$  to about 300  $\mu\text{m}$  into the tissue and are brought in contact with a specific metabolite, wherein a change in fluorescence or absorption (*e.g.*, measured using fluorescence or absorption spectroscopy) of the one or more reporters occurs, thereby allowing quantification of the change in fluorescence or absorption that provides detailed *in vivo* information regarding picomolar through millimolar cellular metabolite and precursor levels for living tissue, organs, interstitial fluid, and whole organisms.

The reporters can be monitored non-invasively using any low-cost instrumentation capable of directly analyzing the metabolic state in tissue (*e.g.*, using optical instrumentation). The reporters are chosen to specifically enhance the signal of pre-specified analytes in order to assess metabolic state of a tissue or organism and to yield detailed, real-time information regarding the state of intracellular and extracellular metabolism.

#### 15 Discussion of Properties Of SMMR Compounds: Physicochemical, Photochemical, Photophysical And Biological

SMMR compounds consist of elements of molecular sub-structure which confer the special properties required to fulfill their specific metabolic reporter function:

1. A fluorescent reporter, with specific photophysical properties,
- 20 2. Chemical functional groups that confer affinity to metabolites, enzymes, cell organelles, membranes, or glucose itself,
3. Structural features that confer specificity between the target of interest and similar targets, which are present in the biological medium (*e.g.*, glucose versus fructose).

The chemistry of small molecule metabolic reporter (SMMR) compounds combines a number of parameters that, in general, results in the following characteristics: SMMR compounds are non-toxic; they have high molar absorption coefficients, and a high quantum yield of fluorescence. They have a large Stokes shift; they are readily taken up by cells, and are retained in the active form at the target tissue. They undergo a large change in fluorescence in response to the metabolism monitored, they are photostable, they do not exhibit excited state chemistry, and they are eventually lost from the body by shedding of the stratum corneum. The molecular design behind these characteristics is the subject of this invention.

Toxicity

The metabolic impact of the compounds to be used as SMMRs is low because of a number of properties that are common to these molecules. When minute quantities may be absorbed into the body they are readily eliminated from the system via biotransformation (metabolism) of the aromatic rings through hydroxylation by a variety of nonspecific enzymes contained within the microsomes of the liver endoplasmic reticulum. Small, more water soluble metabolites result, which are then eliminated from the body by passing through the glomeruli into the proximal tubules of the kidney and into the urine. SMMRs have strict requirements relative to toxicity. Four main criteria must be met: (1) they do not bind to DNA, (2) they do not disrupt cell membranes, (3) they are used at low concentration (*e.g.*, 50 µL of 250 µM), and (4) the SMMR is delivered to a limited volume of tissue, typically the viable epidermis.

The Activity Index (A.I.) of the SMMR compounds is an indication of the effective dose required to elicit an appropriate response to a metabolic signal or glucose. It is indicated by the ratio of the Toxic Dose (T.D.) to the Effective Dose (E.D.) as in equation (1). Note that the A.I. for any proposed SMMR must be greater than 1.0 and ideally should be 10,000 or more.

$$A.I. = \frac{T.D.}{E.D.} \quad (EQ. 1)$$

20

A better indication of the safety of the SMMR would be indicated by the Minimum Activity Index (A.I.<sub>min</sub>) as the ratio of the Maximum Tolerated Dose (T.D.<sub>max</sub>) for 100 percent of the tested group, indicating a maximum dose at which no adverse effects occur, to the Maximum Effective Dose used (E.D.<sub>max</sub>), whereby the maximum signal occurs as equation (2). The larger the A.I.<sub>min</sub> the better toxicity to effective signal characteristics the SMMR possesses. This number must always exceed 1.0 with values greater than 5.0 considered optimum.

25

$$A.I._{min} = \frac{T.D._{max}}{E.D._{max}} \quad (EQ. 2)$$

SMMR molecules with quantum yield ( $\phi_F$ ) values substantially less than unity (e.g., less than 0.3) or those with longer fluorescent lifetimes may show phototoxicity via a photodynamic effect, a process that is unrelated to the inherent toxicity of the SMMR. Phototoxicity arises from reactions of an excited state or from a reactive intermediate generated by the excited state. Phototoxicity can be minimized by using low light doses combined with high quantum yields (e.g., less than 5 mW excitation with a  $\phi_F$  greater than 0.6), thereby decreasing the energy available to form damaging oxygen radicals, and the number of excited states generated, respectively.

As a general rule, exogenous materials such as drugs or, with reference to this embodiment, SMMRs that interact with more than one metabolic pathway have a reduced likelihood of becoming clinically significant due to the availability of compensatory pathways if one is inhibited. Interactions, and hence toxicity, are likely to be increased if the SMMR is an inhibitor or inducer of a particular enzyme, if the response of the SMMR is critically dependent on the concentration and particularly if turnover of the SMMR occurs via a single specific pathway (see for example, Johnson, M. D. *et al.* Clinically significant drug interactions. *Postgraduate Medicine* 1999; 105(2): 193-222).

To a large extent the rationale for SMMR design has avoided many of these problems by targeting normal overall changes in the chemistry of the cell rather than the concentration or activity of a specific metabolite. For example, the monitoring of reducing equivalents within a cell by energy transfer does not affect the overall intracellular concentration of those equivalents nor are there interactions between the enzymes responsible for metabolism and the SMMRs. For some molecules, the toxicity or carcinogenicity is not related to the parent molecule but the metabolite of the molecule. This is often a result of activity of the liver on the molecule. For example, metabolites of benzene are formed by the action of cytochrome P450 in the liver to form, epoxides, phenol, catechol and muconaldehyde. Many of these compounds are extremely reactive and toxic and they may be metabolized further to other toxic materials.

#### Molar absorption coefficient

The molar absorption coefficient ( $\epsilon$ ) of a typical SMMR is high (greater than 50,000 dm<sup>3</sup> mol<sup>-1</sup> cm<sup>-1</sup>). This implies that the probability of a transition from  $S_0 \rightarrow S_1$  is high. The amount of light absorbed is given by the Beer-Lambert law as equation (3).



$$\log \frac{I_0}{I_t} = A = \epsilon cl \quad (\text{EQ. 3})$$

Where,  $I_0$  is the intensity of the incident light,  $I_t$  is the intensity of the transmitted light,  $A$  is the absorbance,  $\epsilon$  is the molar absorption coefficient,  $c$  is the concentration and  $l$  is the pathlength. This expression can also be rearranged to give the fraction of incident light that is absorbed ( $I_a$ ) as described in equation (4).

$$I_a = 1 - 10^{-A} \quad (\text{EQ. 4})$$

For an SMMR concentration of  $10\mu\text{M}$  in a sample thickness of  $100\mu\text{m}$  (typical skin thickness for skin epidermis), a molar absorption coefficient of  $50,000\text{ dm}^3\text{ mol}^{-1}\text{ cm}^{-1}$  results in 11% of the incident light being absorbed. Ultimately, the more light absorbed the more will be converted into fluorescence. In designing an SMMR a high molar absorption coefficient ( $\epsilon$ ) is important; practically this means values greater than  $50,000\text{ dm}^3\text{ mol}^{-1}\text{ cm}^{-1}$ .

The probability of the  $S_0 \rightarrow S_1$  transition occurring can be explained in molecular terms by consideration of the type of bonding that is present in the SMMR. A high probability for the transition requires good overlap between the orbitals in the ground and excited state. Such overlap is found for  $\pi-\pi^*$  transitions and charge transfer states. Transitions involving non-bonding electrons, *i.e.*,  $n-\pi^*$  are not as probable and hence the molar absorption coefficients are lower for these type of transitions. However, the electrons in a non-bonding orbital are higher in energy than electrons in a bonding orbital and therefore the  $n-\pi^*$  transition occurs at lower energy than the  $\pi-\pi^*$  transition.

Because of the low probability of the  $n-\pi^*$  transition the excited state generated by such a transition is expected to be longer lived. Since the balance between the rate constants for radiative and non-radiative decay determines fluorescence quantum yield a long lifetime, in general, allows a greater probability for radiationless decay to occur. Hence,  $n-\pi^*$  transitions tend to be non-fluorescent.

#### Fluorescence quantum yield

Following absorption of a photon the SMMR is promoted to an electronically excited state. The molecule undergoes vibrational changes and interactions with the solvent that result in relaxation to the state from which fluorescence occurs. If this state only undergoes

spontaneous emission then the yield of fluorescence is high ( $\phi_F = 1$ ). If the state undergoes any other process, such as internal conversion or other photophysical change, then  $\phi_F < 1$ . The fluorescence quantum yield ( $\phi_F$ ) of an ideal SMMR is close to unity. Compounds with  $\phi_F$  less than unity are likely to be less photostable and more photoreactive. The factors that are common in the design of an SMMR that result in a high quantum yield are:

1. Rigidity of the molecule. Constraining a molecule limits the number of vibrational modes by which the excited state can be deactivated. Binding of ethidium bromide to DNA for example, increases the quantum yield of the molecule by 30 times.
2. Lack of heavy atom effect. Heavy atom substituents, such as iodine or bromine, cause spin orbit coupling in a molecule and facilitate intersystem crossing. The excited singlet state for such a molecule can readily form a triplet state. Not only does this process decrease the fluorescence quantum yield but it also generates a potentially long-lived reactive state.
3. Bonding character. As mentioned above,  $n-\pi^*$  transitions are, in general, not only weakly absorbing but also non-fluorescent.  $\pi-\pi^*$  transitions have high molar absorption coefficients and are fluorescent. For molecules that have both an electron donating and withdrawing group attached to the  $\pi$  system, the transitions that can occur are described as charge transfer. Typical electron donating groups include amine and hydroxyl groups and withdrawing groups include carbonyl and nitro groups. The transitions are intense and if the transition is of the lowest energy, they are also fluorescent.

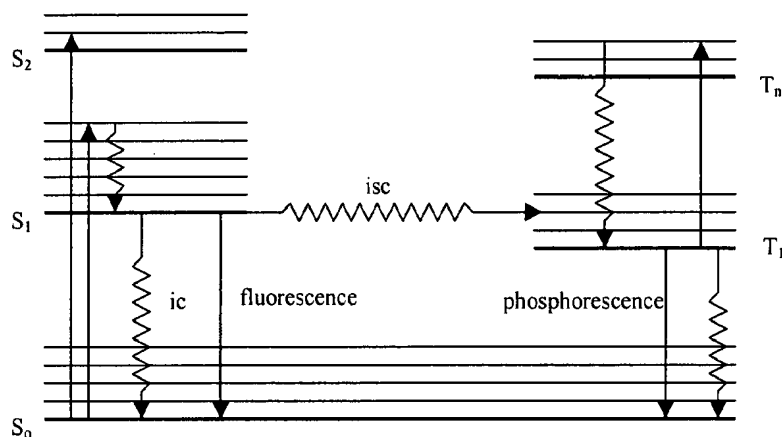
From this discussion, it is apparent that the intensity or brightness of fluorescence is determined by the product of the molar absorption coefficient and the quantum yield ( $\epsilon\phi_F$ ). If brightness is sufficiently high, then even at low concentration, the SMMR absorbs excitation light strongly and efficiently converts this energy to fluorescence. As a general rule then the minimum requirement for an SMMR is that  $\epsilon > 50,000 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  and  $\phi_F > 0.2$ ; thus brightness or  $\epsilon\phi_F \geq 25,000 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ .

#### Stokes shift

The Stokes shift is the difference in energy between the lowest energy absorption and the highest energy emission of a molecule. The advantage in having a large Stokes shift is that

it is much easier, from a practical standpoint, to optically eliminate the influence of the excitation light on the detected light, *i.e.*, the bandpass filter requirements are simplified.

Consideration of a Jablonski diagram (below) would imply that for an  $S_0$  to  $S_1$  transition, involving only the lowest vibrational levels, the energy of the fluorescence should be the same as the absorption. This is almost never precisely the case. The main reasons are threefold:



1. Consideration of the bond order in the transition. In a  $\pi$ - $\pi^*$  transition the electron distribution in the excited state involves non-bonding orbitals. As a result the bond length and the magnitude of the vibration in that bond increases. This process results in a loss of energy and therefore the fluorescent transition occurs from an excited state slightly lower in energy than the state generated by the absorption.
2. The lower bond order in the excited state literally causes the molecule to expand. This change in volume is measurable using photoacoustic spectroscopy. For a molecule in solution to expand requires work to be done in pushing back the solvent. This work results in a loss of energy in the excited state and an increase in the Stokes shift. The volume change increases as the size of the  $\pi$  system increases.
3. The greater the degree of flexibility in the molecule the more the number of vibrational modes available to the molecule. For a complex molecule energy may be lost from parts of the molecule not associated with the chromophore. Any mechanism that causes the molecule to lose energy, including solvent and intermolecular interactions, will lead to a decrease in the energy of the observed fluorescence.

It is noted that factors that serve to increase the magnitude of the Stokes shift also serve to lower the overall fluorescence quantum yield. A novel method to increase the separation of the excitation and emission wavelengths for an SMMR is to covalently link two fluorescent probes together. In practice the molecule that absorbs the light need not even be fluorescent  
 5 provided its excited state lifetime is long enough to transfer energy to the acceptor molecule that fluoresces. This kind of system increases the design flexibility of the SMMR. Proposed examples to monitor pH include a molecule where both the donor and acceptor are sensitive to pH and because of electrostatic changes associated with pH changes, the energy transfer process is also sensitive. ratiometric measurements of the donor and acceptor fluorescence  
 10 show very large changes as a function of pH.

An ideal SMMR has a Stokes shift of about 50 nm or more. Xanthene dyes typically have a Stokes shift of 5 – 15 nm but this value is also dependent on the pKa of the molecule. As an example, BeXan type dyes exhibit Stokes shifts of about 40 nm for the acid form and 60 nm for the basic forms of the dye.

#### 15 Predicting spectroscopic properties from molecular structure

The prediction of spectroscopic properties such as absorption and emission spectra are very difficult. The absorption of a photon involves the promotion of an electron from a ground to excited state. There is currently no molecular calculation algorithm nor automated software package that can take into account the chemical microenvironment of a molecule. The position  
 20 of the  $S_1 \leftarrow S_0$  transition is calculated by considering the energy of the molecule in its ground equilibrium configuration and the energy of the excited state in the same geometry, which is not an equilibrium conformation for the excited state. This is a consequence of the fact that an electronic transition occurs with no change in the geometry of the molecule during the transition (the Born-Oppenheimer approximation). The intensity of the absorption is dependent  
 25 on the probability of the transition. In general  $\pi^* \leftarrow \pi$  transitions are intense and have molar absorption coefficients of  $10^5 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  or greater. High probability is favored by strong overlap between the ground and excited state orbitals.

#### Affinity to target site

Designing SMMR compounds to have an affinity for specified cellular locations,  
 30 membranes, or structures helps reduce noise in an SMMR measurement. When the SMMR is targeted to specific cell locations, both the immediate chemical microenvironment of the SMMR molecule as well as its location for optical measurement can be more closely

controlled. SMMRs can be designed to have high affinity for membranes, organelles, charged structures including specific membrane layers, biopolymers, protein or enzyme binding sites, and regions of the cell that are particularly hydrophobic or hydrophilic. Absolute specificity may be conferred on an SMMR by binding it to a variety of other membrane specific binding  
 5 substituents including the use of antibodies.

Examples of an SMMR structure designed to confer target affinity are given here:

*Membrane affinity:* Amphipathic molecules have a high affinity for membranes. Hydrophilic molecules become amphipathic when an alkyl chain is linked to the structure. This linkage is not necessarily covalent in nature. Electrostatic complexes of cationic detergents and methyl  
 10 viologen, for example, are stable and bind strongly to membranes. In binding to a membrane, the alkyl chain is solubilized in the hydrophobic core of the membrane and the hydrophilic head group is located at the surface of the membrane. This type of molecule is particularly effective at monitoring changes at the interface between the bulk phase and the membrane that include membrane potential and pH changes, either of which may be used to track glucose  
 15 concentration at the cell.

*Enzyme binding site affinity:* SMMR affinity for an enzyme-binding site may be conferred by covalently linking a model enzyme substrate or enzyme cofactor to the SMMR, *e.g.*, as disclosed in pending U.S. Application No. \_\_\_\_\_, entitled "Method for Non-invasive, *in vivo* monitoring of Blood Glucose Levels," filed Jan. 9, 2003, that discusses the binding of an  
 20 SMMR to the FAD cofactor of glucose oxidase.

#### Skin uptake

One of the most important functions of skin is to protect the essential tissues of the body from the outside environment. The layer of skin that forms the physical barrier for the body, preventing moisture loss, infection and regulating temperature is the stratum corneum.  
 25 This cell layer is both hydrophobic as well as acidic, and thus presents a number of problems to transdermal drug delivery. The problem is exacerbated in SMMR technology, since the goal of delivering an SMMR to the epidermis is to have the dye pass through the stratum corneum but to localize in the living epidermal layer. The factors that affect skin uptake include molecular size, hydrophobicity and volatility.

30 The ideal characteristics of an SMMR, useful to penetrate the stratum corneum, include a low molecular weight (less than 600 g mol<sup>-1</sup>), a partition coefficient of about 10, yielding good solubility in lipid and water phases and a low melting point (see for example, "Novel

mechanisms and devices to enable successful transdermal drug delivery,” B.W. Barry. Eur. J. Pharm. Sci. (2001) 14 101-114). A low melting point correlates with high solubility since there is little interaction between the molecules.

For an SMMR that will eventually localize in the cytoplasm or the interstitial fluid, the molecule must have good water solubility. However, a hydrophilic molecule will have difficulty in passing the hydrophobic environment of the stratum corneum. These opposing properties of the molecule imply that the design of the molecule has to be altered to accommodate both environments, that the molecule is amphipathic or that the delivery system can accommodate both polar and non-polar molecules. Delivery systems are customized for individual SMMR properties, and the preparation/formulation of such delivery systems are within the skill of one of ordinary skill in the art, taken with the disclosure herein. It is well known that mixed solvent systems using mixed organic solvents for the organic phase have a significantly greater range of properties. Other solvent systems include the use of surface-active agents to expand the range of properties of the vehicle.

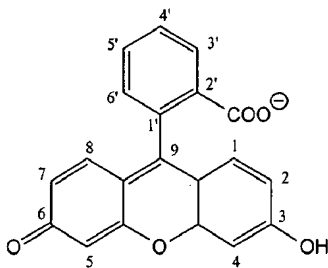
#### 15 Cellular uptake

To a certain extent the factors that affect uptake in the skin are the same as those that affect cellular uptake. Overall, there has to be a balance between movement of the SMMR into the stratum corneum, diffusion into the epidermis, and then the competing processes of SMMR uptake by the epidermal cells and loss of the dye into the dermis. All of these processes may be considered to be reversible. The most important factors that facilitate cellular uptake of a small molecule include the partition coefficient (P), and the molecular size and the diffusion coefficient in the lipid matrix (D). Diffusion across a biological membrane is dependent on three factors: the diffusion of the SMMR from the aqueous phase to the lipid phase, diffusion within the lipid phase and diffusion from the lipid phase back into an aqueous phase. For a molecule whose flux across the membrane is proportional to the concentration gradient the proportionality constant is called the permeability coefficient (C<sub>p</sub>) and it is directly dependent on the three factors above. The permeability coefficient is given by the expression in equation (5).

$$C_p = \frac{DP}{x} \quad (\text{EQ. 5})$$

Where  $x$  is the distance across the membrane, or more accurately the distance across the concentration gradient, the other terms are as defined above. For most SMMRs the permeability is directly proportional to the partition coefficient although this relationship does not hold for very small molecules.

- 5        The  $pK_a$  is important, since this will determine the overall charge of the molecule at a particular pH and hence the partition coefficient. For many pH indicating xanthene compounds there are two  $pK_a$  values of importance. As an example, fluorescein



- will be considered. The  $pK_a$  of fluorescein is 6.4. Below this pH, the hydroxyl group drawn at position 3 is protonated. In practice, the oxygen atoms at positions 3 and 6 are equivalent, and above pH 6.4 the xanthene ring is negatively charged with electron density shared between these two positions. The carboxylic acid at the 2' position is unprotonated until the pH drops to about pH 4.2. Below a pH of 2.2, the carboxylic acid ring closes at position 9, there is a hydrogen shift onto the carbonyl at position 6 and the quinoid structure no longer exists. Under these conditions the compound is colorless. The relevance of this parameter to drug delivery in the skin is that this type of compound displays many of the characteristics that facilitate SMMR delivery.

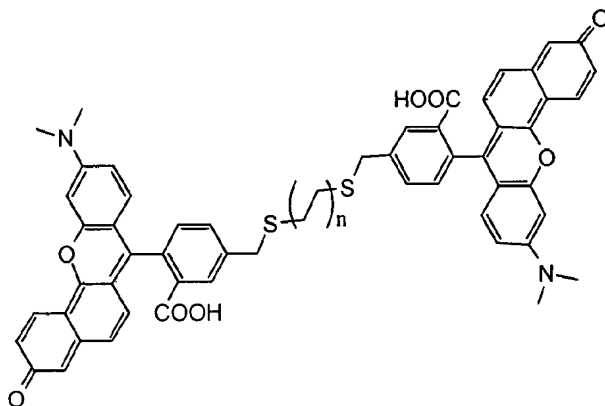
- SMMRs designed with acidic functionality have greater potential for skin and cellular uptake. In skin, the stratum corneum is acidic and the surface of cell membranes is also acidic. Therefore the use of SMMRs with acidic substitutions result in a significant fraction of compound molecules being uncharged at pH 5 (close to the values reported in the stratum corneum and at the cell surface). These molecules are therefore more hydrophobic, may cross the barrier and once inside the cell, at  $\sim$  pH 7 become deprotonated again. Suitable groups include carboxylic acids ( $pK_a \sim 4-5$ ) and aromatic thiols ( $pK_a \sim 8$ ). Both of these groups are electrostatically neutral when protonated.

Cellular uptake and retention may be improved by the use of polyoxyethylene chains or polylysine chains. The purpose of these substitutions are to give the SMMRs both hydrophobic

and hydrophilic properties. The molecule behaves as a cross-linked micelle changing its conformation and degree of order depending on its environment. The chromophore itself is hydrophilic but it can be solubilized in non-polar environments by the long alkyl chain. In a hydrophobic environment, the chain wraps around the molecule and, if a poly-oxaethylene is used the chain is configured to present the oxygen atoms of the chain towards the interior of the complex. In a hydrophilic environment, the chain unwraps and the SMMR is exposed.

SMMR species connected by flexible saturated chains behave as individual chromophores when located in environments where the monomer is soluble. Under conditions where the monomer is not soluble, the dimer will fold to present the smallest volume of the molecule to the environment. This approach has previously been used to deliver photodynamic therapeutic (PDT) agents to tissues. The folded dimer, which is often non-fluorescent, may cross the cell membrane and unfold when it reaches its cytoplasm related target. The unfolded dimer is again photochemically active.

An example of a dimeric BeXan type molecule is shown below:



It is readily synthesized from the chloromethyl derivative of the monomer reacting with a dithioalkane. Thioethers are known to be biologically stable molecules (*see, e.g.*, Effect of linker variation on the stability, potency, and efficacy of carcinoma-reactive BR64-doxorubicin immunoconjugates. P.A. Trail, D. Willner, J. Knipe, A.J. Henderson, S.J. Lasch, M.E. Zoeckler, M.D. TrailSmith, T.W. Doyle, H.D. King, A.M. Casazza, G.R. Braslawsky, J. Brown, S.J. Hofstead, R.S. Greenfield, R.A. Firestone, K. Mosure, K.F. Kadow, M.B. Yang, K.E. Hellstrom and I. Hellstrom. *Cancer Research*, (1997) 57(1) 100-105; and Enhancing selectivity, stability, and bioavailability of peptidomimetic estrogen receptor modulators F.



Spatola, A. K. Galande, F. M. Brunel, K. S. Bramlett, and T. P. Burris, Presented at the 18<sup>th</sup> American Peptide Symposium, July 19-23, 2003, Boston, MA).

### Cellular retention

The compounds used as SMMRs must be retained inside the cell so that repeated applications are not necessary for an SMMR monitoring device to function over extended periods from 1 hour to 30 days. Many of the same factors that determine how well an SMMR will cross the membrane also determine whether the compound will leak out of the cell. The principal factors that influence compound retention include: charge, size, polarity, pKa, and the presence of groups that interact with cellular components. Assuming that the principal mechanism for leakage out of the cell is diffusion across the membrane, the SMMR compound should be large, hydrophilic and preferably negatively charged to prevent leakage.

Molecules with molecular weights of greater than 600 g mol<sup>-1</sup> will be retained to a much higher degree than smaller molecules. Negatively charged species are electrostatically repelled from cell membranes. Cell membranes have a high pH gradient near the surface. Negatively charged surfaces attract cationic species, which include the highly mobile hydrogen ion, in a layer called the Stern layer. This layer, located near the membrane surface, may be as much as two pH units lower than the phase of the bulk membrane. Therefore, to maintain charge in the vicinity of a membrane, it is important that at least some of the protonatable groups on the molecule have pKa values less than pH 5.

Quantitatively, leakage from the cell may be measured by monitoring the fluorescence of the interstitial fluid or medium in which the cells are bathed. The leakage is dependent on the concentration gradient and therefore leakage will be higher in a cell culture type measurement than in a skin type system. For example, if a 35 mm diameter dish confluent with keratinocytes and bathed with 1 ml of medium is compared to the same number of cells stacked as the cells in the epidermis, then the apparent leakage rate would be at least 30 times higher in the cell culture system. The leakage rate may ultimately be compared to the target site affinity (T.S.A.) parameter (equation 3):

$$\text{T.S.A.} = \frac{\text{SMMR}_{\text{active}}}{\text{SMMR}_{\text{delivered}}} \quad (\text{EQ. 3})$$

The T.S.A. value is related not only to the leakage rate but also to the rate at which the SMMR is metabolized or photobleached within the cell. These are the same parameters that would have prevented the compound entering the cell initially. Therefore, ideally the SMMR

should be converted to this type of molecule after it has entered the cell. This conversion has been accomplished in a number of ways.

Esterification of an SMMR leads to a hydrophobic molecule that can cross the cell membrane. Once inside the cell, the ester is cleaved off by esterase enzymes, generating a charged molecule that cannot readily pass back out of the cell across the membrane. Reduced compounds such as dihydrotetramines are hydrophobic and may be oxidized inside the cell to form the fluorescent, hydrophilic form of the compound. Other methods that have been used include substitution of a chloromethyl group that interacts with thiol groups leading to conjugation with proteins or hydrophilic moieties preventing leakage from the cell.

The affinity of the SMMR to the target tissue is also given as the Target Site Affinity (T.S.A.). The T.S.A. indicates the percent of SMMR that remains active at the target site after physical delivery to the site. It is reported as a time dependent phenomenon relative to 1 hour, 24 hours, and 72 hours. The T.S.A. for any time period is given in equation (3) as the ratio of moles of SMMR delivered to moles of SMMR active at the delivery site.

#### Metabolic monitoring (indirect glucose measurement)

SMMR technology is designed to specifically target metabolic pathways. For SMMRs designed to track glucose these pathways have a direct relationship with the *in vivo* glucose concentration. For the most part biological pathways do not stand in isolation from other processes that occur in the body. It is therefore possible to improve the sensitivity of monitoring by targeting more than one pathway at the same time. For example, it is useful to know the percentage of metabolism that occurs by oxidative phosphorylation, and the fraction that occurs by anaerobic metabolism. This knowledge allows different cell types or cells under different conditions to be compared. Increasing the number of pathways monitored increases the specificity of the measurements, the dynamic range (since measurements can be made under a wider array of conditions) and decreases the influence of competing processes. As an example of the use of SMMR technology to monitor glucose concentration, the pathways that would be targeted are shown in Figure 1. The technique to monitor each pathway is given.

SMMR technology is able to monitor glucose transport through the use of membrane bound reporters that respond to the activity of the glucose transporter molecule (GluT). It has previously been shown that the kinetics of GluT may be monitored from the autofluorescence

of tryptophan residues in the protein. SMMR technology can monitor the GluT protein either by energy transfer from tryptophan to the dye, or by monitoring membrane dynamics in the vicinity of the GluT protein.

Under conditions where there is excess glucose, cells can convert glucose to glycogen.

5 Glycogen is stored within the cytoplasm of cells as small granules. The size of these granules is fairly uniform and is on the order of tens of nanometers in diameter. As the amount of glycogen stored increases the number of granules increases not the size of the granules. Glycogen synthesis is measured in tissue biopsies using the absorption of the glycogen:iodine complex at 460 nm. Thus, this aspect of the glucose metabolism pathway is measurable using  
10 optical means.

In tissue that undergoes primarily anaerobic metabolism, the products of the glycolysis reaction pathway are lactate and adenosine triphosphate (ATP). ATP is synthesized from ADP, the diphosphate analog, and inorganic phosphate. Lactate is generated as a waste product of the pathway. The lactate concentration within the cell is dependent on lactate transport out of  
15 the cell and the rate of glycolysis. The extracellular lactate concentration is dependent on lactate transport and diffusion of lactate into the blood stream. Published work has correlated the production of lactate with intracellular pH. Both intra and extracellular pH is measured using SMMR technology with ratiometric monitoring. To monitor the pH values simultaneously, dyes with different spectroscopic properties are used. To use SMMRs with  
20 overlapping spectra requires the SMMRs to be applied to different regions of the skin and then repetitive measurements to be made at each site.

#### Photostability

The photostability of a fluorophore is a function of the magnitude of the quantum yield. If the excited state of the SMMR undergoes any process other than radiative or decay via a  
25 vibrational cascade the possibility for a photochemical reaction to take place and an attendant loss of photostability. The loss of fluorescence is the result of a photochemical reaction, often involving the excited state of the compound, and the generation of a photoproduct. This process is generally called photobleaching, which means literally the loss of color. Photobleaching is often an oxidation process and the degree of photobleaching may be  
30 proportional to the number of excited states generated. Therefore, photobleaching can be minimized by using low intensity excitation light, a low oxygen concentration and by increasing antioxidant concentrations.

Excited state chemistry

The processes that can lead to a photochemical reaction include: energy transfer from the excited singlet state ( $S_1$ ), electron transfer from  $S_1$  energy transfer from the excited triplet state ( $T_1$ ), electron transfer from  $T_1$  formation and subsequent reaction of singlet oxygen

5 ( $O_2(^1\Delta_g)$ ). These reactions are summarized in the following Figure (2) where:

- SMMR +  $h\nu$  represents the absorption of a photon
- $^1\text{SMMR}^*$  is the first excited singlet state
- $^3\text{SMMR}^*$  is the first excited triplet state
- S represents some biological substrate
- 10  $+ \bullet$  and  $\bullet -$  represent a semioxidized and semireduced species respectively
- $O_2(^1\Delta_g)$  is singlet oxygen
- i.s.c is intersystem crossing.

The k terms in the diagram are the rate constants for each process.  $E_{lt}$  and  $E_{nt}$  refer to  
15 electron transfer and energy transfer respectively. For an SMMR to have a high quantum yield the rate constant for fluorescence has to compete with all of these processes.

Turnover

The turnover of the SMMR is related to a number of factors including: photostability,  
20 localization, metabolic activity involving reaction with the SMMR, leakage out of cells, uptake into the blood stream, migration into the stratum corneum and loss to the environment. Some SMMR turnover is an advantage since the process reduces the potential for a photobleached compound or compounds to migrate into a non-active region of the tissue. Turnover due to photochemical effects has been discussed earlier.

25

SMMR REPORTING ACTIVITY

The activity of an SMMR is dependent on its response to the metabolic pathway to which it is targeted as well as its ability to reach the site of that pathway. The chemical properties of the compound that determine its potency include pKa, excited state energy levels,  
30  $\phi_F$ ,  $\epsilon$ , octanol:water partition coefficient, and the selectivity of the SMMR for the targeted pathway.

The design of a suitable SMMR involves the correlation of the chemical properties of the SMMR with the biological reporting activity of the compound. To be able to do this it is critical that the reporting activity of the compound be quantified so that different compounds can be compared and a correlation derived.

5           There are several parameters that can be determined to measure the efficacy of an SMMR. These parameters include: minimum concentration that can be detected using fluorescence, smallest change in analyte concentration that results in a measurable spectroscopic change, and dynamic range in the SMMR response

10           In a series of papers published in the 1960's, Hansch and co-workers described how certain aspects of a drug structure could be related to its activity. (Comparison of parameters currently used in the study of structure-activity relationships. A. Leo, C. Hansch and C. Church. *J. Med. Chem.* (1969) 12(5) 766-771; Homolytic constants in the correlation of chloramphenicol structure with activity. C. Hansch, E. Kutter and A. Leo. *J. Med. Chem.* (1969) 12(5) 746-749; Passive permeation of organic compounds through biological tissue: a  
15 non-steady-state theory. J.T. Penniston, L. Beckett, D.L. Bentley and C. Hansch. *Mol. Pharmacol.* (1969) 5(4) 333-341; The linear free-energy relationship between partition coefficients and the binding and conformational perturbation of macromolecules by small organic compounds. F. Helmer, K. Kiehs, and C. Hansch. *Biochemistry*. 1968 7(8) 2858-2863; Correlation of ratios of drug metabolism by microsomal subfractions with partition  
20 coefficients. E.J. Lien and C. Hansch. *J. Pharm. Sci.* (1968) 57(6) 1027-1028.) In particular, the hydrophobicity of a molecule described how it could partition between tissue and bodily fluid.

A similar approach for SMMR design may be used to provide a semi-empirical approach to SMMR design. Two examples are given, one for an SMMR that monitors a  
25 biological pathway, such as glycolysis, via a change in intracellular pH; and one for an SMMR that is used to monitor a biological pathway, such as glycolysis, via the overall reduction potential of the cell through energy transfer.

*SMMR reporting glycolysis via a change in intracellular pH*

30           Using an analogous rationale to that described by Hansch, an empirical equation (4) that would allow the prediction of the smallest concentration of an SMMR that could be detected from its fluorescence following application to the skin is provided as follows.

$$\log \left( \frac{1}{[C]} \right) = k_1 \log A \phi_F + k_2 \log P - k_3 (\log P)^2 + k_4 pK_a + k_5 \quad (\text{EQ. 4})$$

Where:

C is the smallest concentration that is detectable in the skin,

5 A is the absorbance of the solution,

$\phi_F$  is the quantum yield of the compound,

P is the octanol water coefficient,

$pK_a$  is the  $pK_a$  of the compound,

the constants  $k_1$  through  $k_5$  are empirically determined constants obtained through

10 linear regression.

The determination of the unknown parameters in this equation requires that at least five times the number of observations be made, as there are terms in the equation. In the example given here at least twenty-five observations would have to be made.

15 The term  $k_1 \log A \phi_F$  describes the probability of the SMMR absorbing a photon and reemitting it as fluorescence. The higher the absorbance and the quantum yield, the more likely is the absorption of a photon and the generation of fluorescence. The term  $k_2 \log P - k_3 (\log P)^2$  describes the partition between hydrophobic and hydrophilic phases within an organism. P is the octanol water partition coefficient and has been shown to describe the  
 20 distribution of a solute between the bulk aqueous phase and the hydrophobic phase of a lipid bilayer such as a cell membrane. The optimum value of P is some intermediate value. Hydrophilic molecules remain in the bulk phase while hydrophobic molecules are not solubilized and therefore are not carried to the cell membrane.

The  $pK_a$  is included in this equation because it is related to the partition coefficient of  
 25 the SMMR. Protonation of basic groups and deprotonation of acidic groups lead to an increase of charge in the molecule and hence increased hydrophilicity. Equation (5) is a relatively simple equation that merely describes the factors that control the uptake of a molecule into a cell membrane. For an SMMR to be effective, the molecule must be retained in the cell or tissue. To a certain extent, retention is described by the same factors that describe uptake of

the compound. An equation that describes the response of an SMMR to a change in pH caused by the activity of a metabolic pathway is given here:

$$\log R = k_w \log \left( \frac{A_{\phi_F}^{DH}}{A_{\phi_F}^D} \right) + k_x pK_a + k_y \log P + k_z \quad (\text{EQ. 5})$$

5 Where:

R is the difference in the fluorescence under the extreme conditions of the metabolic pathway, maximum and minimum activity.

The superscript DH and D refer to the protonated and deprotonated forms of the compound respectively.

10

All other terms are as in equation (1) and the constants  $k_w$  through  $k_z$  are determined empirically as before. Essentially the first two terms in this equation form the Henderson-Hasselbalch equation (Die Berechnung der Wasserstoffzahl des Blutes auf der freien und gebundenen Kohlensäure desselben, und die Sauerstoffbindung des Blutes als Funktion der Wasserstoffzahl. K.A. Hasselbalch. Biochem. Z. (1916) 78, 112-144). The log P term appears because the SMMR must localize in a similar region of the cell as the location of the biochemical pathway.

15

Further discussion of the design concept, and compounds and related constructs are described in the following examples.

20

### EXAMPLE 1

#### Using pH to track D-glucose concentration in living cells

For human keratinocytes, the carefully measured intracellular pH (as a measure of lactate production) is directly proportional to the concentration of D-glucose entering the cell.

25

Thus, a decrease of intracellular pH is indicative of an increase in glucose concentration. The lactate formation within the cell is in direct proportion to the quantity of glycolysis occurring within the cell, and this glycolysis is 'fueled' by D-glucose and other simple sugars, such as fructose and galactose. This example demonstrates the protocol for precise pH measurement within viable cells, which is directly related to the D-glucose concentration within viable

30

human keratinocytes.

A-431 cells obtained from ATCC (#CRL-1555) are seeded at  $5 \times 10^5$  cells in 35mm culture dishes (Falcon #353801) containing a #2 25mm cover glass (VWR#48382-085). Cells are incubated in 2 mL Dulbecco's Modified Eagle's Medium at 100mg/dL D-glucose (Gibco #11966-025) at 90% and Fetal Bovine Serum (Gibco#26140-087) at 10%. Cells are allowed to reach near confluence in 6% CO<sub>2</sub> 37°C incubator, over a period of 3 to 5 days.

The glass cover slip with cells is dipped and rinsed in Dulbecco's Phosphate-Buffered Saline (D-PBS) (*Invitrogen*, Catalog # 14040) or pH 7.2 HEPES buffer with composition (mM): NaCl 150, KCl 4.5, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 1.8. The cover slip is then mounted slip on a Sykes-Moore Chamber (#1943-11111, *Belco Glass*, Vineland, NJ) to form a study chamber.

The chamber is always sat on a hot plate at  $36.5 \pm 0.5$  °C. A-431 cells are washed three times with the buffer. After wash, 1 ml buffer solution with 1 mM (18 mg/dL) D-glucose is added into the chamber. The background spectrum of A431 cells in buffer solution is then measured by in-house developed clinical development breadboard (CBB).

A ratiometric pH reporting dye is used as the *in vitro* intracellular pH indicator. 1 mM stock of the ratiometric pH reporting dye in DMSO is prepared and stored under -20°C. Cells are loaded in the presence of  $0.4 \sim 2$  μM of the dye in HEPES buffers (pH should be calibrated to 7.22 at 35°C), plus 1 mM D-glucose under 36~37°C for 1 hour. During the time, the cell chamber is shaken gently three times for homogenous loading. The cell is then washed four times with same buffer solution. Washing solution should be kept in the cell chamber for 3 minutes and then decanted. After washing, 1 ml buffer solution with 1 mM D-glucose is added into the cell chamber.

Two cell chambers are required for a complete study. One cell line is for pH change from neutral to acidic and the other is for the change to basic. About 12 minutes after dye loading, 5 spectra of cell chamber 1 are measured on the CBB as intracellular pH measurements. The whole solution is next replaced by 1 ml buffer solution with 15 μM nigericin (15 μL of 10 mM nigericin), and 5 spectra of pH 7.22 are measured 3 to 5 minutes later. For pH changes from neutral to pH 6, 10 μL pH 1.18 HEPES buffer is added continuously into 1 mL solution contains cells and nigericin. Each addition provides about 0.2 pH unit decrease. Five spectra at different pH are measured about 3 to 5 minutes after adding 10 μL pH 1.2 HEPES buffer. The procedure is repeated about 5 times. For measurements at pH 5, 2.5 uL 1 M HCl in distilled water is added into the chamber.



For the pH measurement from neutral to basic, the same procedure as used for intracellular pH and pH 7.22 are measured on cell chamber 2. For pH changes from neutral to pH 8.2, 10  $\mu$ L pH 12.3 HEPES buffer is added continuously into 1 mL solution containing cells and nigericin. Each addition provides an increase of approximately 0.2 pH unit. Five spectra  
 5 at different pH are measured about 3~5 minutes after adding 10  $\mu$ L pH 12.3 HEPES buffer. The procedure is repeated about 4 times. For pH 9 measurements, 5  $\mu$ L 1 M NaOH in DW is added in the chamber. The exact pH of 1 ml 7.22 HEPES buffer by adding certain amount of pH 1.2 or pH12.3 buffer should be calibrated by pH meter.

The basic assumption for the Henderson-Hasselbalch model to apply is: the  
 10 fluorescence intensity of each form (protonated and deprotonated) of the dye is linearly proportional to that form's concentrations. The application of the modified model, the quantum yield and photobleaching of two forms should not be affected differently by environmental effects.

The governing equation for this model is

15

$$\text{pH} = \text{pK}_a - \log \left[ \frac{r - r_B}{r_A - r} \times \frac{F_{\lambda_2}^B}{F_{\lambda_2}^A} \right] \quad (\text{EQ. 6})$$

where  $r$  is the ratio of fluorescence intensity at  $\lambda_1$  to that at  $\lambda_2$ ,  $r_A$  is the ratio for the fluorescence of the protonated form,  $r_B$  is the ratio for the deprotonated form, and  $F_{\lambda_2}^{A,B}$  is the  
 20 fluorescence intensity of the protonated and deprotonated forms, respectively, at  $\lambda_2$ . The  $\text{pK}_a$  in Equation 1 is true  $\text{pK}$  and it can be expressed as

$$\text{pK}_a = \text{pK}_{\text{app}} + \log \left[ \frac{F_{\lambda_2}^B}{F_{\lambda_2}^A} \right] \quad (\text{EQ. 7})$$

25 where  $\text{pK}_{\text{app}}$  is the apparent  $\text{pK}$  at the ratio of  $\lambda_1$  over  $\lambda_2$ .

The ratiometric pH reporting dye has a pale color in dimethyl sulfoxide (DMSO). Aqueous solution of the ratiometric pH reporting dye will actually exhibit different visible colors at pH 8 to 9. The absorption spectra of the ratiometric pH reporting dye (5  $\mu$ M in 0.1 M

phosphate buffer) are measured on an HP 8453 UV/Vis spectrometer, an example of which is shown in Figure 3. Fluorescence emission spectra excited at 532 nm were measured on a Fluorolog® spectrometer (Jobin Yvon Inc., 3880 Park Avenue, Edison, NJ 08820-3012 USA) and data are shown in Figure 4. Samples were prepared from a series of combinations of 0.1 M monobasic sodium phosphate and 0.1 M dibasic sodium phosphate. Both sodium phosphate solutions contain the ratiometric pH reporting dye at 5  $\mu$ M.

For the SMMR dynamic study, 12 minutes after SMMR loading, five spectra of one cell line are measured at 1 spec/min. Preheated (in water bath) 10  $\mu$ L 10 g/dL D-glucose or same volume of control solution is added to the chamber. Twenty five spectra are measured after solution adding. The first 10 are at 30-second intervals, and then change to 1 minute. A set of cell lines (4 to 6) is followed above procedure by adding D-glucose and control solution.

For the statistical study, 12 minutes after dye loading, with a 30 second gap, half of total (~8) cell lines are added with 10  $\mu$ L D-glucose, and another half with 10  $\mu$ L control solution. Several rounds of measurements of all cell lines are carried out at appropriate intervals. The difference between adding D-glucose and control solution (L-glucose or buffer) will be studied statistically. The cell viability is assessed and recorded using fluorescence light microscopy.

## EXAMPLE 2

### Using external, in vivo pH measurement to track blood glucose or blood lactate

The rationale for measuring blood glucose levels or blood lactate levels using external, *in vivo* optical measurements of SMMR activity within skin is demonstrated. Fluorescence measurements *in vivo* of SMMR placed within the skin during glucose clamp studies were designed to improve the observation of the correlation between glucose levels and measured pH changes.

After improvements of fluorescence measurements, additional clamp studies with better control over anesthesia were done to demonstrate the consistency and reliability for the correlation between glucose levels in blood and the reporter dye fluorescence ratio.

This description demonstrates the results of additional glucose clamp studies during which fluorescence signals were measured *in vivo* after dye injections using low-cost components comprising a fluorescence sensing device.

An example ratiometric pH reporting dye was prepared as described by diluting a 1 mM stock aliquot (frozen at  $-20^{\circ}\text{C}$  in DMSO). The final concentration typically used for placement into the skin was 20  $\mu\text{M}$  in PBS. The SMMR was delivered by shallow injection (or topocal passive diffusion) into hairless rats using 100  $\mu\text{L}$  of solution. Multi-injections at the same location were repeated 4 times at intervals of 1 hour. Clamps were started on the next day (15 – 20 hours after last injection) after the preliminary pre-heating of the rat, *i.e.*, rat had laid on heated stage about 20 minutes before measurements. During bottom measurement of the rat the average temperature of the stage was about  $36.5^{\circ}\text{C}$ , and the standard deviation of the stage temperature during a single experiment did not exceed  $0.5^{\circ}\text{C}$ .

During this study the influence of anesthesia was minimized. Results of *in vivo* measurements are shown as in Figures 5-8.

Changes of intensity were not substantial during the experiments. Anesthesia (0.19 cc of ketamine) was administered by injection to each rat 75 minutes before measurements. Relative changes in time of parameter  $U$  can be defined as  $100 [U(t)-U(0)] / U(0)$ , where  $t$  is time (in minutes), and 0 denotes time at the beginning of measurements. As noted from Figure 1d, observed relative changes for glucose and lactate are substantially greater than the corresponding changes of the fluorescence ratio. To provide a more meaningful visual comparison between glucose, lactate and fluorescence ratio changes, a normalization expression of the independent data are used as:  $(U-U_{\min}/U_{\max}-U_{\min})$ , which refers to the difference between given value of ratio, lactate, or glucose and its minimum value divided by the difference between its maximum value and its minimum value. Figure 9 shows the normalized and relative changes for the independent data sets over time: fluorescence ratio, lactate, and glucose.

The experimental data allows estimating the sensitivity coefficients of ratio change to glucose change and lactate change as given in equation (8).

$$\Delta R = \alpha \Delta G + \beta \Delta L \quad (\text{EQ. 8})$$

Noting that the expression,  $\Delta R/\Delta G = 5 \times 10^{-4} \text{ dL/mg}$  and  $\Delta R/\Delta L = 2 \times 10^{-2} \text{ dL/mM}$ , where:  $L$  has units  $\text{mM/dL}$  and  $G$  has units  $\text{mg/dL}$ .

Previous clamps with anesthesia (0.1 cc of ketamine) during measurement provided estimates of the average kinetic changes in fluorescence ratio, which are shown in Figure 10.

Taking into account the influence of three major factors, namely: anesthesia, lactate and glucose it is possible to compare experimentally observed changes during clamp studies with calculated values using an expression such as equation (9):

$$\Delta R = \alpha \Delta G + \beta \Delta L + \Delta R_a(t-t_a) A \quad (\text{EQ. 9})$$

5

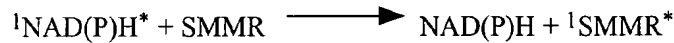
Where  $t_a$  is the moment of time when anesthesia was administered;  $\Delta R_a(t-t_a)$  is the experimentally defined ratio changes due to anesthesia with 0.1 cc of ketamine; and A is amount of ketamine (in 0.1 cc units).

Figures 11a – c show these results. From these results, the positive correlation between the fluorescence SMMR ratio measurements and changes in glucose and lactate are demonstrated.

#### SMMR operating through energy transfer

Assuming that SMMRs can be delivered to the sites at which energy transfer takes place (as described by equation (4) above, then a new expression can be derived for the efficiency of an SMMR monitoring the overall reduction potential of the cell by energy transfer, *i.e.*,

20



In this process, the excited state of the reduced nicotinamide interacts with the SMMR to generate the excited state. The  $\phi_F$  of NAD(P)H is less than 0.1. If an SMMR is chosen with a quantum yield close to unity, then the yield of fluorescence is increased by at least one order of magnitude, provided the energy transfer process is efficient, equation (10).

25

$$\log F = k_a \log \frac{k_T}{(\tau_D^{-1} + k_T)} + k_b \log P + k_c \quad (\text{EQ. 10})$$

Where:

F is the fluorescence response to the change in intracellular reduction potential,

$k_T$  is the rate of energy transfer,

$\tau_D^{-1}$  is the reciprocal of the fluorescence lifetime,

30

P is the partition coefficient,

and  $k_a$ ,  $k_b$  and  $k_c$  are the determined empirical constants as described previously.

The rate constant of energy transfer is given by the expression in equation (11).

5

$$k_T = \left( \frac{\phi_F \kappa^2}{\tau r^6 C n^4} \right) J(\lambda) \quad (\text{EQ. 11})$$

Where:

$\kappa$  is known as the orientation factor,

10  $\tau$  is the fluorescence lifetime of the donor,

$\phi_F$  is the fluorescence quantum yield of the donor,

C is a collection of constants,

$J(\lambda)$  is known as the overlap integral,

and n is the refractive index of the medium.

15

The orientation factor describes how the transition dipoles of the donor and acceptor molecules align. For two molecules moving randomly in solution, the value of  $\kappa^2$  is about 0.66, and for two dipoles perpendicular to each other the value is 0.

The term  $k_T / (\tau_D^{-1} + k_T)$  in equation (6) is the efficiency of energy transfer. In words,

20 it is the number of energy transfer events as a fraction of all decay events. The energy transfer rate constant must be significantly greater than the sum of all the rate constants attributed to all other decay routes for the energy transfer to be efficient.

The log P (or log  $P_{o/w}$ ) value reflects the relative solubility of any drug in octanol (representing the lipid bilayer of a cell membrane) and water (the matrix fluid within the cell  
25 and in blood).

**EXAMPLE 3**Using NADH Fluorescence to track D-glucose concentration in living cells

A set of demonstration experiments for living cells has shown the expected trend in NADH signals with respect to a change in glucose concentration, as shown in Figure 12 (glucose from 0 to 400 mg/dL) and Figure 13 (glucose from 0 to 66 mg/dL). All glucose concentrations above ~ 5mg/dL were well above the saturation limit as can be seen by the lack of any further change in signals.

A second set of demonstration experiments was designed to determine the saturation limit by varying glucose concentration from 0 to 5 mg/dL and back to zero. The saturation point is expected to have a value between 0.0 and 2 mg/dL.) As shown in Figure 14 an approximate linear increase in NADH fluorescence is observed with glucose concentration from 0 to 5 mg/dL (0.28 mM). Figure 15 is a plot of a background subtracted NADH signal where the background changed linearly as determined from the first zero glucose signal and the last (near) zero glucose signal (sequence #11). Figure 16 shows the NADH signal trend versus concentration for living cells.

**EXAMPLE 4**Using Membrane Potential to track D-glucose concentration in living cells

A set of demonstration experiments showed the expected trend in Rh123 fluorescence quenching. The fluorescence quenching is an indication of membrane potential changes within living cells with respect to an increase in glucose concentration, as shown in Figures 17 (glucose from 0 to 400 mg/dL) and Figure 18 (glucose from 0 to 66 mg/dL).

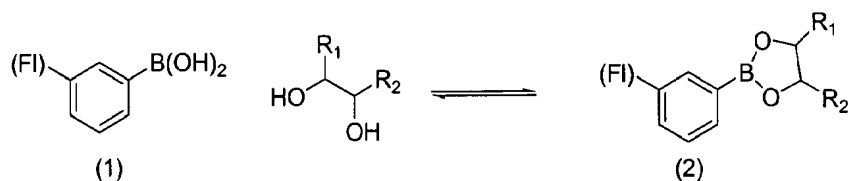
A second set of demonstration experiments was designed to determine the saturation limit for membrane potential-based glucose analysis by varying glucose 0 to 5 mg/dL and back to zero. The saturation point value was expected to be between 0.0 and 2 mg/dL. As shown in Figure 19 an approximate linear decrease in Rh123 fluorescence with increasing glucose levels was observed from 0 to 5 mg/dL (0.28 mM).

SMMR fluorophore-reporter for glucose and diol measurement

For this application of direct glucose sensing SMMRs, the objective is to detect and quantify glucose via a small molecule fluorescent reporter whose photophysical properties are modulated by binding with D-glucose or other simple sugars or diol molecules. Such SMMRs

report glucose using a reversible binding process and the molecular structure-activity consists of three mechanistic parts.

- 1) A fluorophore with suitable photochemical characteristics;
  - 2) A chemical affinity group that binds reversibly with glucose and similar molecular species (*e.g.*, a boronic acid – containing component);
  - 3) Additional substructural features to favor specificity for glucose over fructose, galactose, and other biologically active saccharides, which may be physiologically present near target cells (*i.e.*, near the intercellular or interstitial spaces of the viable epidermis).
- Boronic acids are frequently used in saccharide reporter molecules to provide affinity. Boronic acids undergo reversible binding with glucose or another sugar or diol-containing molecule to form a boronate ester with high affinity. (Scheme 3):

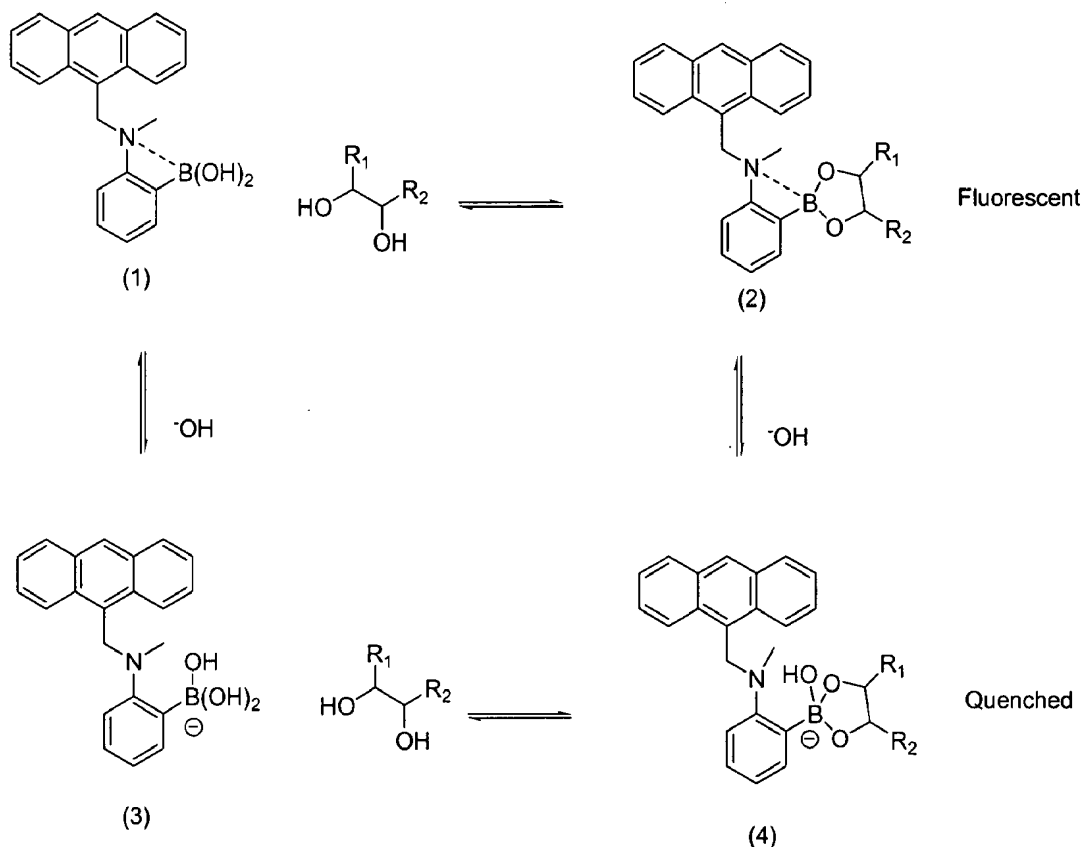


In the literature, signal transduction of the boronic acid + sugar-binding phenomenon has been accomplished in two ways:

- 1) Modulation of Photoinduced Electron Transfer (PET) quenching, and
- 2) Modulation of Internal Charge Transfer (ICT).

The PET mechanism of signal transduction is illustrated schematically in Scheme 4, in the case of an anthracene fluorophore [See for example, T. D. James, K.R.A. Samankumara Sandanayake, R. Iguchi, and S. Shinkai, "Novel Saccharide-Photoinduced Electron Transfer Sensors Based on the Interaction of Boronic Acid and Amine", *J. Amer. Chem. Soc.*, **117**, 8982 (1995)]. In this example, the fluorophore-boronic acid is present in a pH dependent equilibrium, represented by species (1) and (3). For this example, as pH is increased, the fraction of (3) present increases. In this form, the electrons of the nitrogen lone pair are available for fluorescence quenching of the anthracene moiety. At lower pH, the boron atom participates in a coordinate covalent bond, making the nitrogen lone pair electrons unavailable for quenching, resulting in higher fluorescent intensity.

The presence of a sugar molecule perturbs the (1) - (3) equilibrium. The boronate ester (2) exhibits a different pKa from boronic acid (1). Thus, at a given pH the fluorescent-to-quenched state equilibrium is shifted, resulting in a net increase or decrease of observed fluorescence intensity, depending on the pH of the experiment and the relative pKa of the boronic acids (1) and (2)

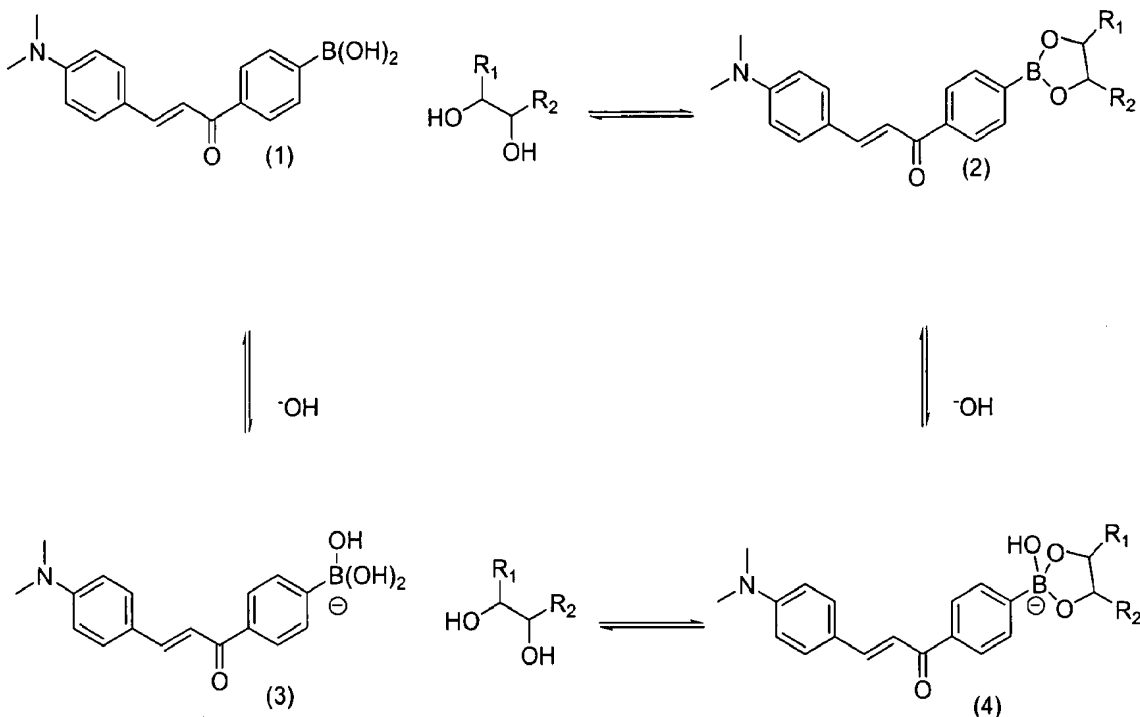


**Scheme 4.** Illustrative example of the PET mechanism of Boronic acid signal transduction in the presence of a saccharide molecule. (T. D. James, K.R.A. Samankumara Sandanayake, R. Iguchi, and S. Shinkai, "Novel Saccharide-Photoinduced Electron Transfer Sensors Based on the Interaction of Boronic Acid and Amine", J. Amer. Chem. Soc., 117, 8982 (1995)).

The ICT mechanism of signal transduction is illustrated schematically in Scheme 5. In this instance the pH dependent equilibrium (1) – (3) is perturbed by the presence of a sugar molecule. At a given value of pH, this results in a different proportion of the fluorophores (boronic acids and boronate esters) being present in the hydroxylated form, thus modulating the electron-withdrawing characteristics of that end of the molecule. In the case of the boronic



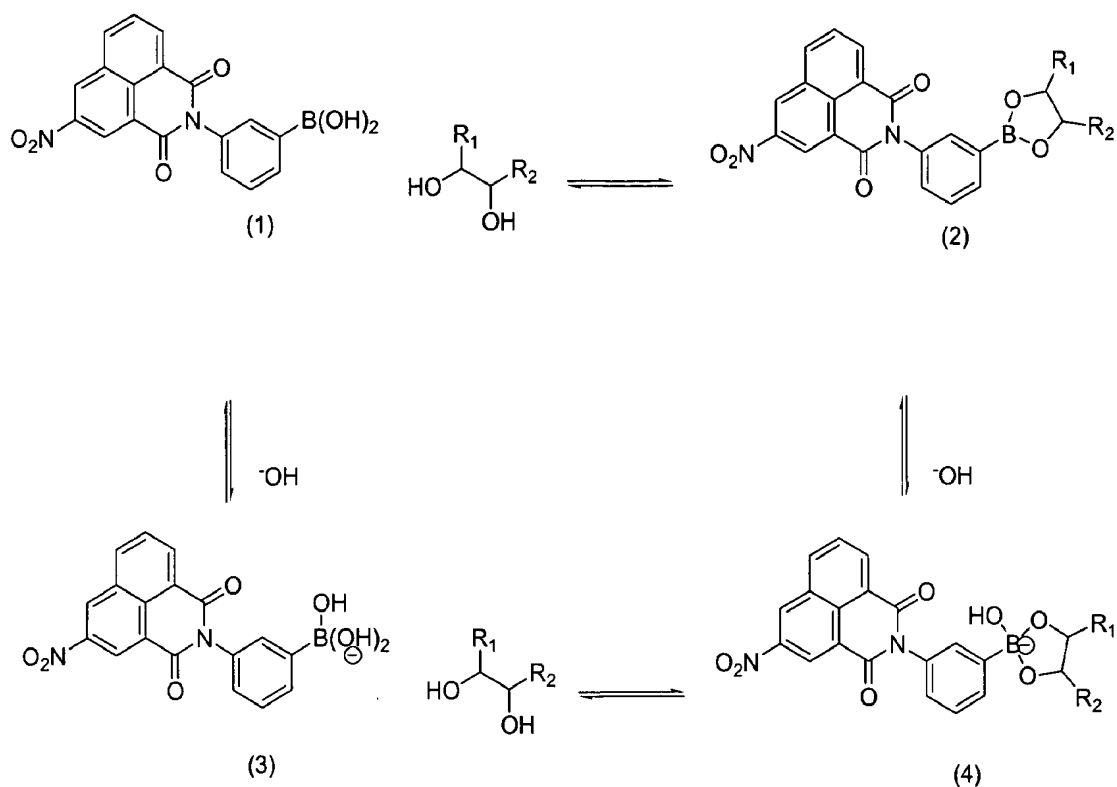
chalcone molecule (1), in Scheme 5, this is manifested as a change in relative fluorescent intensity and only a slight wavelength shift.



5

**Scheme 5.** Illustration of the ICT mechanism of Boronic Acid fluorescent signal transduction. (N. DiCesare and J. R. Lakowicz, "Chalcone-Analog Fluorescent Probes For Saccharides Signaling Using the Boronic Acid Group", *Tetrahedron Letters*, **43**, 2615 (2002); and H. Cao, D. I. Diaz, N. DiCesare, J.R.Lakowicz, and M. D. Heagy, "Monoboronic Acid Sensor That Displays Anomalous Fluorescence Sensitivity to Glucose", *Organic Letters*, **4**, 1503 (2002)).

Another example of the ICT mechanism from Lakowicz's laboratory is shown in Scheme 6. In the case of the N-phenyl-3-nitro-1,8-naphthalimide, the ICT phenomenon manifests itself in a change in relative fluorescent intensity at two wavelengths, resulting in a ratiometric probe. The starting point for these authors' design was the knowledge that suitable N-phenylnaphthalimides exhibit fluorescent emission at two wavelengths and that the longer wavelength emission band shows solvatochromic behavior.



- 5 **Scheme 6.** Schematic illustration of the ICT mechanism (N. DiCesare and J. R. Lakowicz, "Chalcone-Analog Fluorescent Probes For Saccharides Signaling Using the Boronic Acid Group," *Tetrahedron Letters*, **43**, 2615 (2002); and H. Cao, D. I. Diaz, N. DiCesare, J.R.Lakowicz, and M. D. Heagy, "Monoboronic Acid Sensor That Displays Anomalous Fluorescence Sensitivity to Glucose", *Organic Letters*, **4**, 1503 (2002)).

10

#### Designing the Fluorophore-Boronic acid reporter

- This compound consists of a fluorophore with long wavelength excitation and emission and high quantum yield. The phenylboronic acid moiety is incorporated in the molecular structure as an essential requirement to add binding affinity for glucose and other diol-containing compounds. Solvatochromic properties may be defined. The photochemistry of the fluorophore is sensitive to its microchemical environment and solvent polarity. Boronic acid can directly affect photochemical properties such as quantum yield and wavelength if it is attached to the fluorophore and serves as the e-withdrawing portion of a "push-pull" system.
- 20 This can be direct (when boron is connected directly to the fluorophore), or indirect (when the phenylboronic acid exerts a "through-bonds" electron-withdrawing inductive effect).

Prosthetic group selection is critical for fine-tuning molecular selectivity. Naturally occurring enzyme active sites and receptors exhibit exquisite selectivity, even among closely-related compounds. This is possible due to the receptor's high degree of spatial and electrostatic complementarity relative to the molecule in question.

5            In the case of glucose-binding proteins, for example, the selectivity of the enzyme glucose oxidase is achieved by hydrogen-bonding and close contacts between a glucose molecule and more than six amino-acid side chains present in the active site.

             In the realm of small-molecule synthetic glucose reporters, some reported compounds achieve a relatively good specificity for glucose over fructose—as much as 10-fold,  
10        considering that there is very little in the way of molecular features (of the synthetic receptor) to distinguish one from the other. When designing a synthetic receptor, several strategies can be employed to improve specificity, namely:

1. Multiple boronic acid binding sites

             Spatial disposition and distance will provide some selectivity among competing  
15        saccharide molecules, due to geometric constraints. (T. D. James, K.R.A. Samankumara Sandanayake, R. Iguchi, and S. Shinkai, “Novel Saccharide-Photoinduced Electron Transfer Sensors Based on the Interaction of Boronic Acid and Amine”, J. Amer. Chem. Soc., 117, 8982 (1995)). Using two or more boronic acid binding complicates the picture due to the high affinity of the reversible formation of covalent bonds in the  
20        boronate ester. The equilibrium-binding constant of a single boronic acid based receptor is in the millimolar (mM) range for glucose. The corresponding binding constant is micromolar ( $\mu\text{M}$ ) in the two-boronic acid case. Thus, at normal physiological concentrations, the two-boronic probe would be saturated.

25        2. Adjacent H-bond donors and acceptors

             Additional H-bond donors and acceptors contribute only about 6 kcal/Mol., each, to  
ligand-receptor binding. Design of a synthetic probe to include H-bonding sites provides a means to provide a binding advantage to molecules, which present H-bondable groups in the appropriate spatial orientation. Thus the position and  
30        orientation can be designed in by reference to molecular models, to enhance glucose specificity relative to other saccharides.

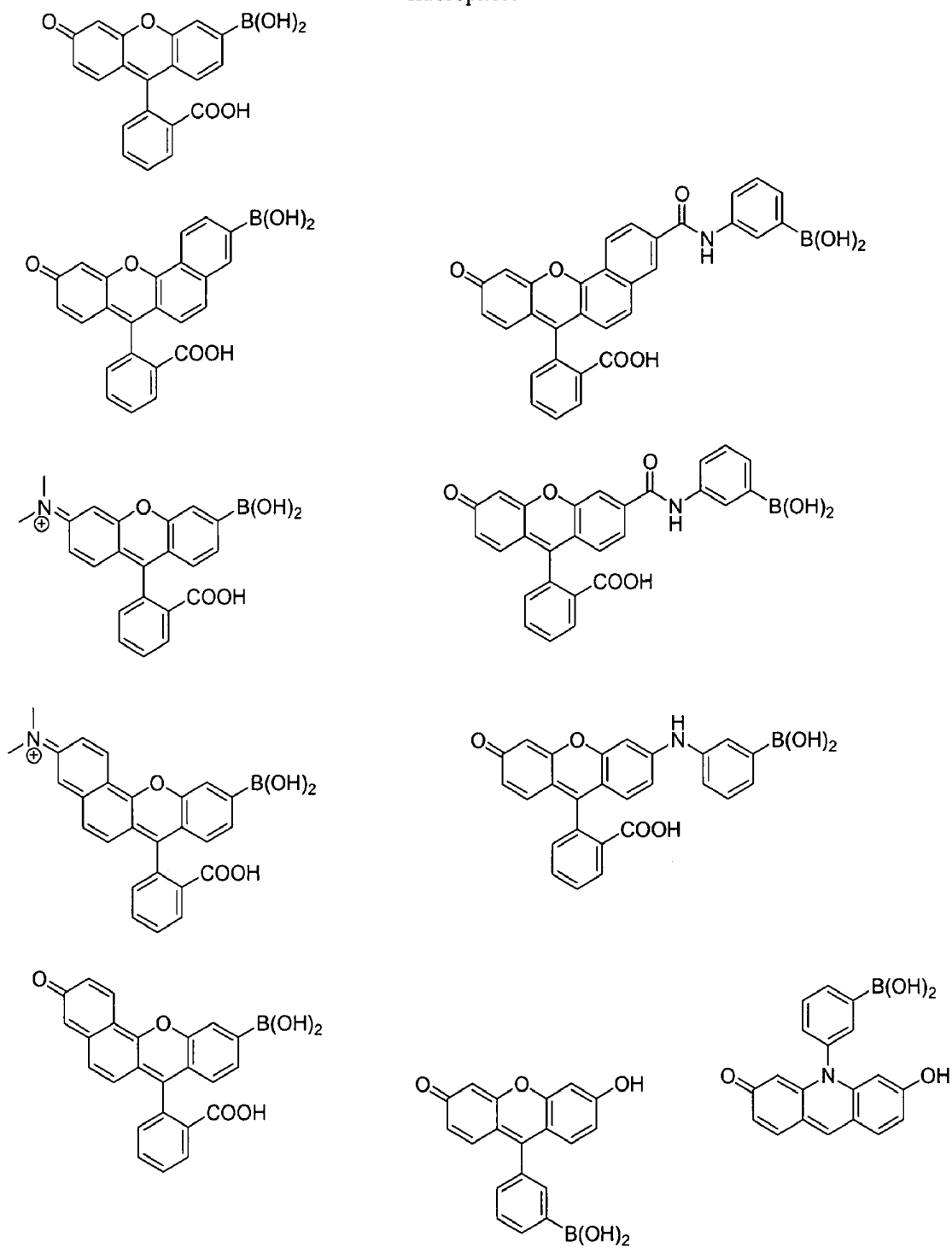
3. Adjacent non-bonded interactions

Specificity can be enhanced by model driven choice of other chemical groups, such as alkyl side chains, which do not add to binding affinity, but operate by spatial exclusion.

- 5            Several examples of novel boronic acid compounds based on various fluorophores are given in the following examples.

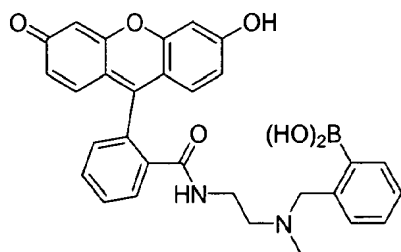
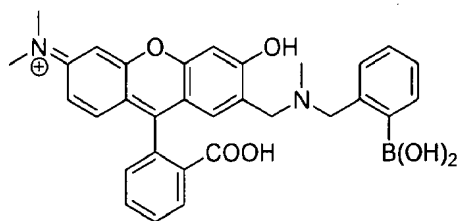
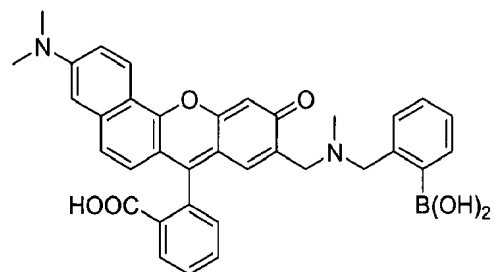
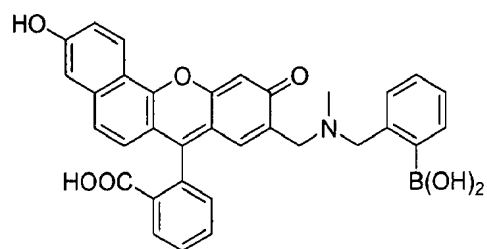
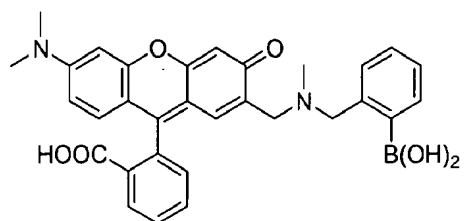
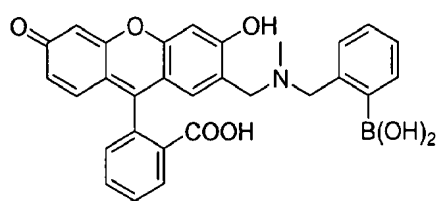
## EXAMPLE 5

Xanthene dyes used to report glucose by the ICT mechanism or electronic perturbation of the fluorophore



## EXAMPLE 6

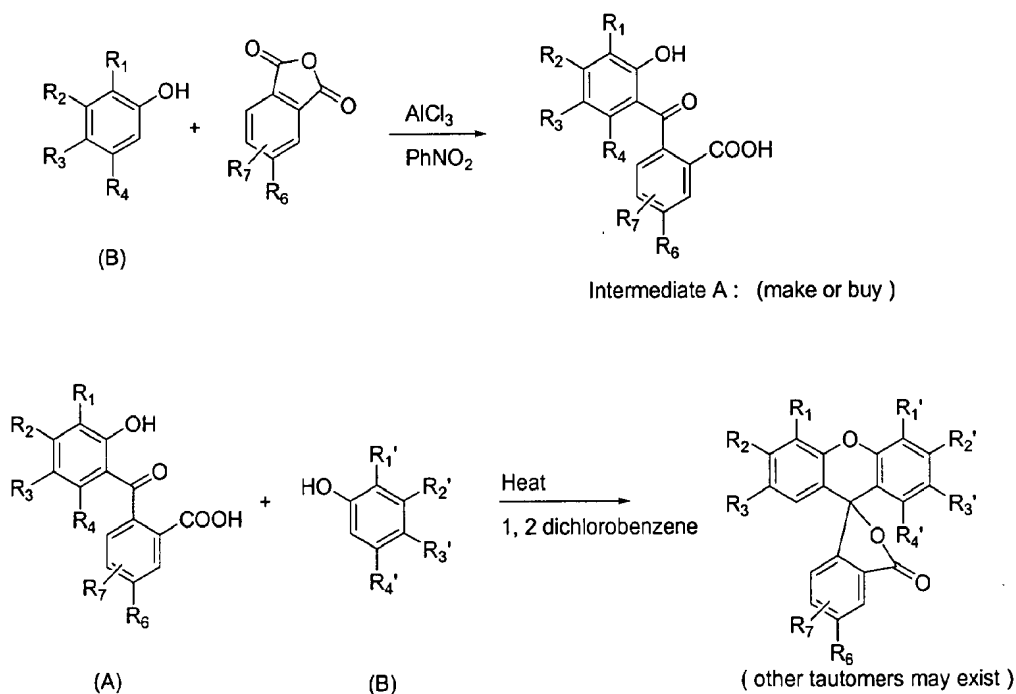
Xanthene dyes used to report glucose by the PET mechanism



Designing xanthene-based reporters

A library of xanthene dyes is proposed to form a basis for Structure-Activity studies with respect to emission wavelength, ratiometric behavior, and quantum yield. The library of compounds is made in a combinatorial synthesis paradigm, wherein each xanthene compound is a result of a cross-product of suitable building blocks. Substituents are chosen with emphasis on final products that incorporate electron-donating and electron-withdrawing groups as well as H-bond donors and acceptors. Thus the library members will include compounds with push-pull characteristics and solvatochromic sensitivity

The library compounds are generated according to the reaction sequence outlined in Scheme 7, below. These reactions employ standard chemical methods. The final reaction yields the nominal product and byproducts corresponding to disproportionation and recombination of the building blocks. Final products are isolated after standard purification techniques such as flash chromatography and semi-preparative HPLC.



**Scheme 7.** A method for the synthesis of a Xanthene Combinatorial Library

The possible products comprising this library are delineated schematically in Figure 20 and the accompanying Tables 1, 2, and 3 as demonstrated.

Tabulated substituents on Building Blocks used in the Xanthene Library.

5

**Table 1.** Xanthene Building Blocks Incorporating R1, R2, R3, R4, and X

Building Block	R1	R2	R3	R4	X
1		OH			O
2		N(CH <sub>3</sub> ) <sub>2</sub>			O
3		OH			S
4		SH			S
5		SH			O
6		OCH <sub>3</sub>			O
7		CF <sub>3</sub>			O
8	OCH <sub>3</sub>	OH			O
9	NH <sub>2</sub>	OH			O
10	NO <sub>2</sub>	OH			O
11	CF <sub>3</sub>	OH			O
12	CH <sub>3</sub>	OH			O
13	Cl	OH			O
14	CN	OH			O
15	CH <sub>3</sub>	OH		CH <sub>3</sub>	O
16	Br				O
17	COOH				O
18		NO <sub>2</sub>			O
19		OCH <sub>3</sub>			O
20		OH		CH <sub>3</sub>	O
21		OAc			O
22		N-Me <sub>2</sub>			O
23		NH <sub>2</sub>			O

**Table 2.** Xanthene Building Blocks incorporating R5, R6, and R7

10

Building Block	R5	R6	R7		
1	COOH	-	-		
2	COOH	-	COOH		
3	-	OH	-		
4	CH <sub>3</sub>	NHAc			
5	-	OH	-		



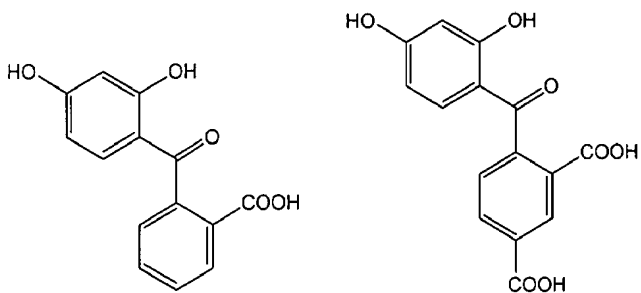
*Table 3.* Xanthene Building Blocks Incorporating R8, R9, R10, R11, and X

Building Block	R8	R9	R10	R11	X
1	OH	-	-	-	O
2	-	OH	-	-	O
3	-	-	OH	-	O
4	-	-	N-Me <sub>2</sub>	-	O
5	-	-	-	Br	O
6	-	-	-	COOH	O

**Appendix 1.** List of building blocks for the Xanthene Library composition

**A Components**

5. **Fluoresceins**

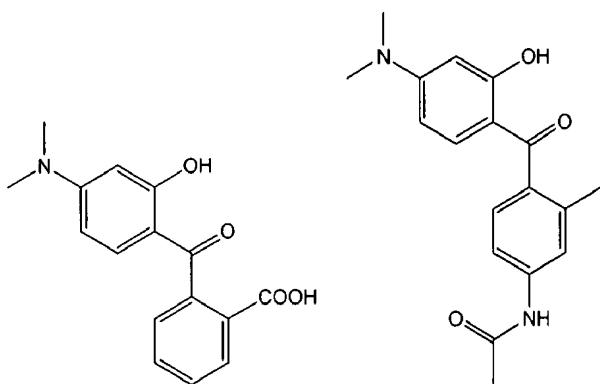


AF1

AF2

10

**Rhodamines**

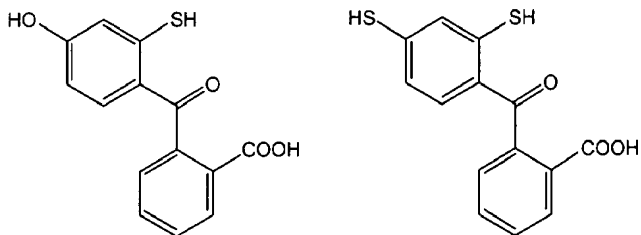


AR1

AR2

15

**Thio derivatives**

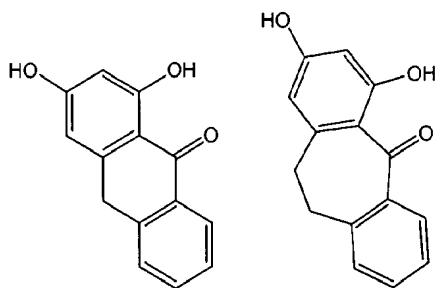


*AT1*

*AT2*

5

*Rigidified xanthenes*



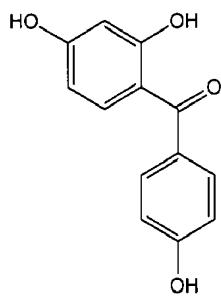
10

*ARg1*

*ARg2*

*Quinoid chromophores*

15

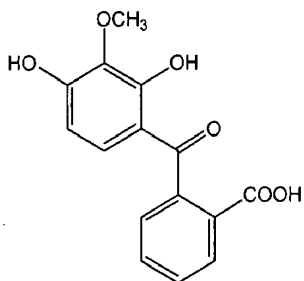


*AQ1*

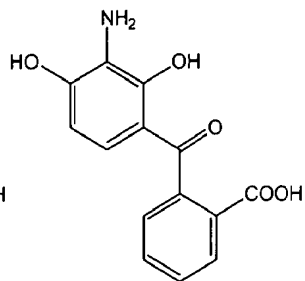
20

Push – Pull xanthenes

**Push**



*APus1*

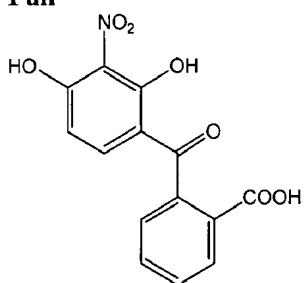


*APus2*

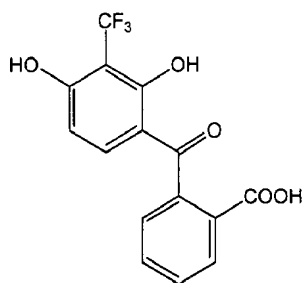
5

10

**Pull**



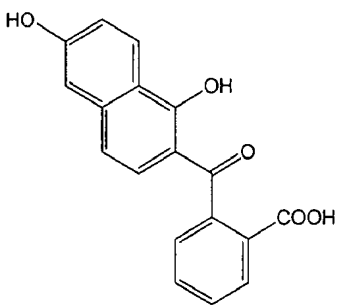
*APul1*



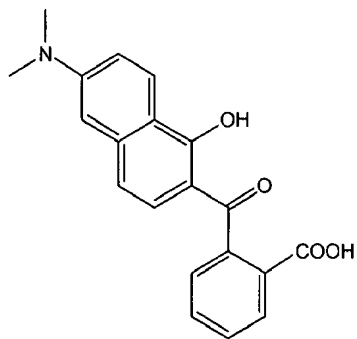
*APul2*

15

Naphtho xanthenes



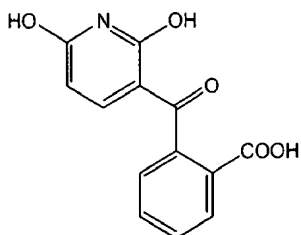
*AN1*



*AN2*

20

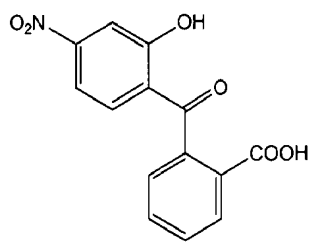
Miscellaneous



5

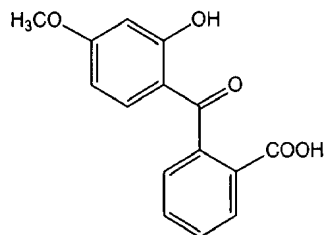
*AM1*

Xanthene analogs



10

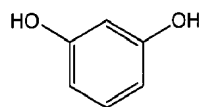
*AX1*



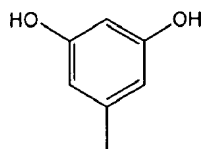
*AX2*

B Components

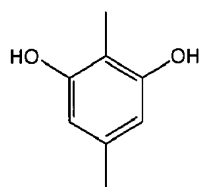
15 Fluoresceins



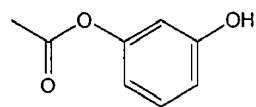
*BF1*



*BF2*



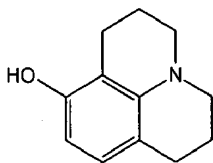
*BF3*



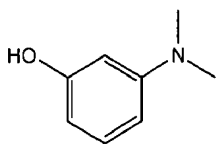
*BF4*

20

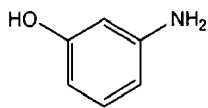
Rhodamines



BR1



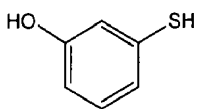
BR2



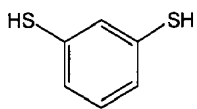
BR3

5

Thiol derivatives



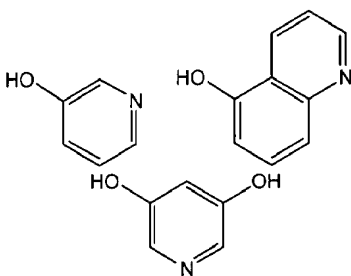
BT1



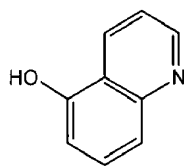
BT2

10

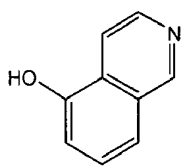
Heterocyclic analogs



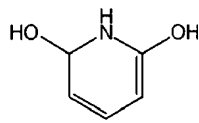
BN1



BN2



BN3



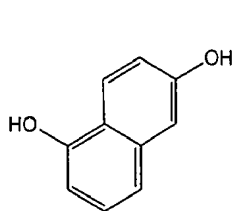
BN4

BN5

15

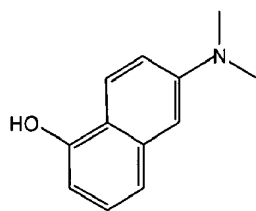
20

Naphtho xanthenes



5

*BNap1*



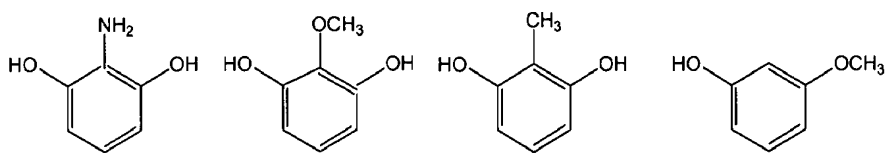
*BNap2*

10

Push – Pull derivatives

**Push**

5



*BPus1*

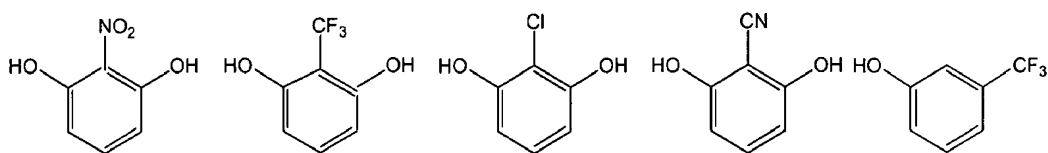
*BPus2*

*BPus3*

*BPus4*

**Pull**

10



*BPul1*

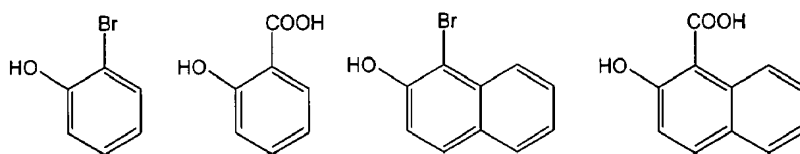
*BPul2*

*BPul3*

*BPul4*

*BPul5*

15 Other



*BM1*

*BM2*

*BM3*

*BM4*



Summary of design strategy

This list comprises 15 A components, 18 B components. Combination of these materials would result in 270 xanthene dyes.

5

The list of components is

*A Components*

	<b>Fluoresceins:</b>	<b>AF1; AF2</b>
	<b>Rhodamines:</b>	<b>AR1</b>
10	<b>Thio derivatives:</b>	<b>AT1; AT2</b>
	<b>Rigidified xanthenes:</b>	<b>ARg1; ARg2</b>
	<b>Quinoid chromophores:</b>	<b>AQ1</b>
	<b>Push – Pull xanthenes</b>	
	<b>Push:</b>	<b>APus; APus2</b>
15	<b>Pull:</b>	<b>APul1; APul2</b>
	<b>Naphtho xanthenes:</b>	<b>AN1; AN2</b>
	<b>Miscellaneous:</b>	<b>AM1</b>
	<b>Xanthene analogs:</b>	<b>AX1; AX2</b>

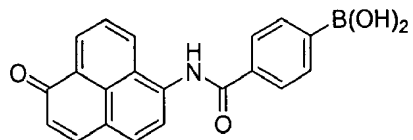
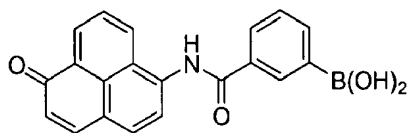
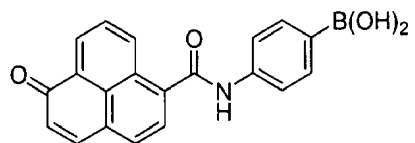
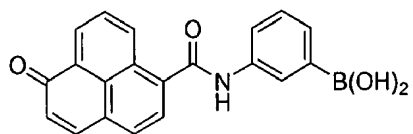
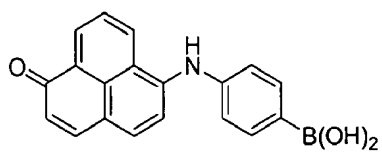
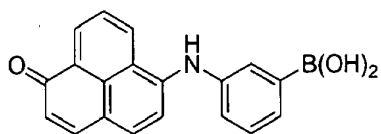
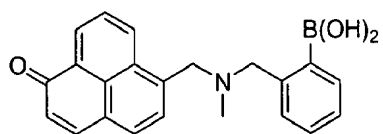
20 *B Components*

	<b>Fluoresceins:</b>	<b>BF1</b>
	<b>Rhodamines:</b>	<b>BR1; BR2; BR3</b>
	<b>Thiol derivatives:</b>	<b>BT1; BT2</b>
	<b>Heterocyclic analogs:</b>	<b>BN1; BN2; BN3</b>
25	<b>Naphtho xanthenes:</b>	<b>BNap1; BNap2</b>
	<b>Push – Pull derivatives</b>	
	<b>Push:</b>	<b>BPus1; BPus2</b>
	<b>Pull:</b>	<b>BPul1; BPul2</b>
	<b>Other:</b>	<b>BM1; BM2; BM3; BM4</b>

30

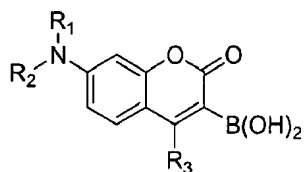
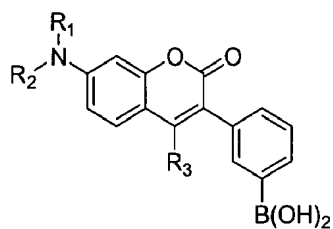
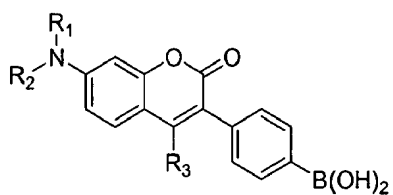
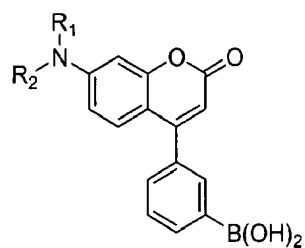
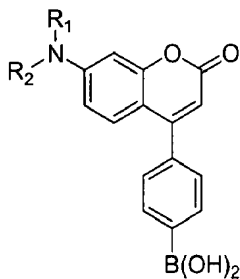
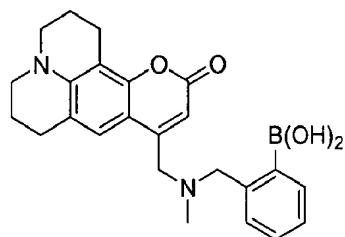
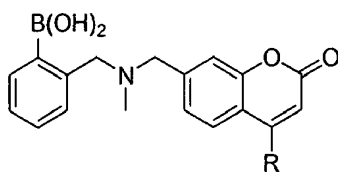
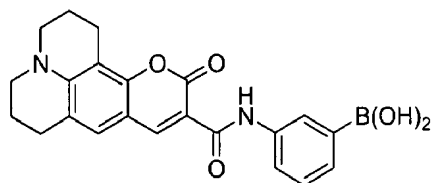
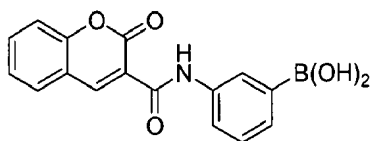
# EXAMPLE 7

Phenylene-1-one dyes used as glucose reporters



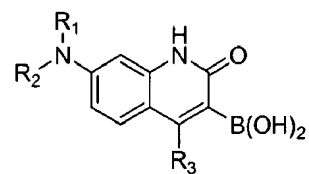
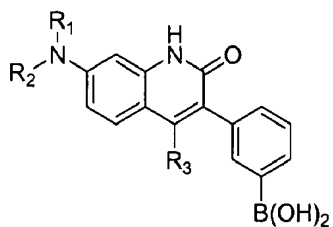
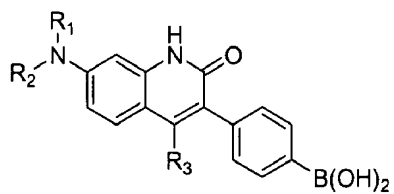
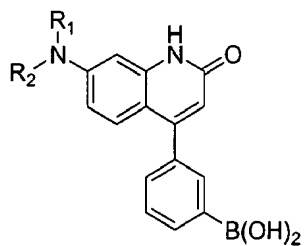
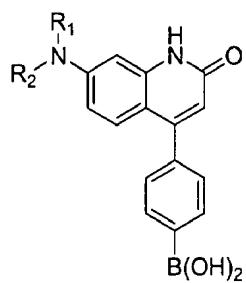
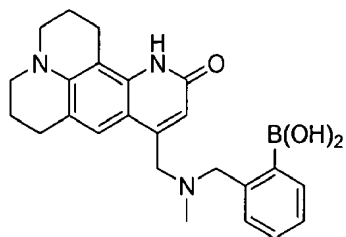
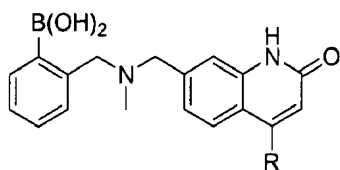
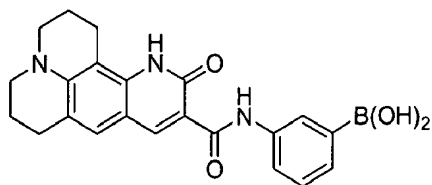
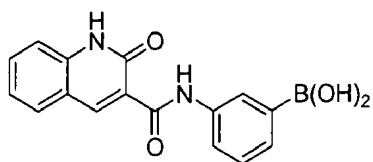
**EXAMPLE 8**

Coumarin derivatives used as glucose reporters



## EXAMPLE 9

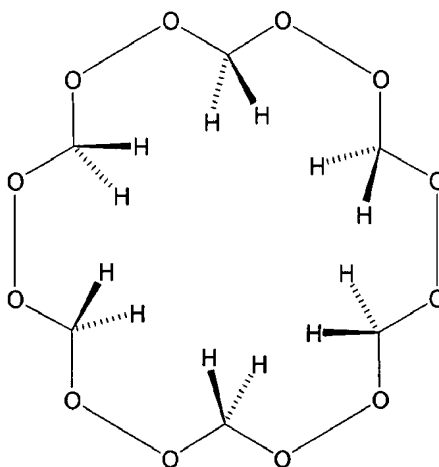
Carbostyryl derivatives used as glucose reporters



**EXAMPLE 10**

## Use of tethered molecules for glucose measurement

- Suitable tethers include both ring structures and linear systems (example given in Scheme 8, below). The advantage of a ring system is that the structure is semi-rigid which limits the degree of entropy in the system. If the system is too flexible, the two chromophores will be less likely to approach each other. In order of complexity, cyclic tethers include cycloalkanes, crown ethers, cyclodextrins and cyclic peptides. The number of residues in the ring system is chosen so that the chromophores come close enough together to promote complex formation.



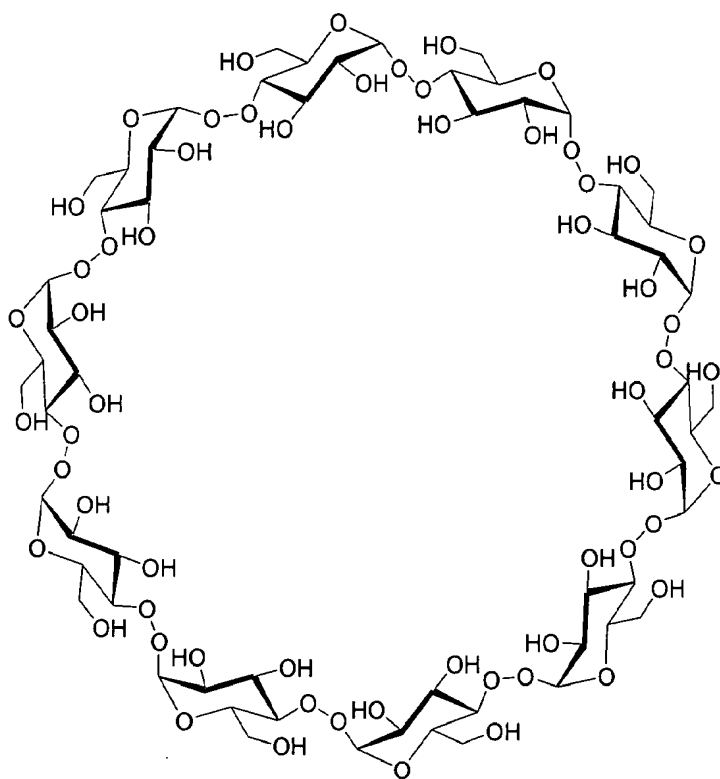
15

Scheme 8. *An example of a cyclic tethered system*

**EXAMPLE 11**

## Use of a crown ether for glucose detection

- Substitution of the ring system may at a ring carbon position for cycloalkanes and
- 5 crown ethers, at a hydroxyl group in cyclodextrins or on any of the amino acid residues in a cyclic peptide (example given in Scheme 9). The cyclic peptide may be designed to have some specificity for glucose by choosing a peptide sequence that mimics the binding site found in a protein such as glucose oxidase or glucose dehydrogenase.



*Scheme 9.* A ten saccharide cyclodextrin

**EXAMPLE 12**

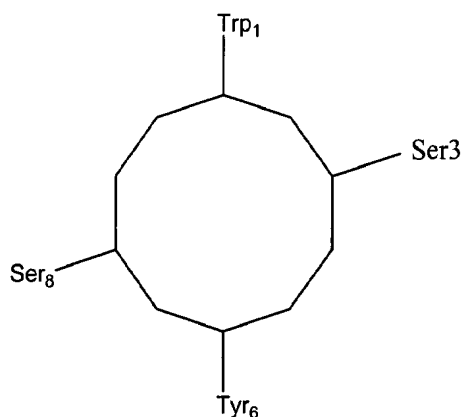
Use of cyclic decapeptide fluorophores for glucose and other diol sensing

This example describes a molecule for use in the detection and quantification of glucose. This glucose sensing occurs by means of a fluorescent reporter whose photophysical properties are modulated by direct binding with glucose or other diol molecules. Such reporters consist of three mechanistic parts:

1. A fluorophore with suitable photochemical characteristics;
2. A chemical affinity group that binds reversibly with glucose and similar molecular species (cyclic peptide);
3. Additional substructural features to favor specificity for Glucose over Fructose and other saccharides.

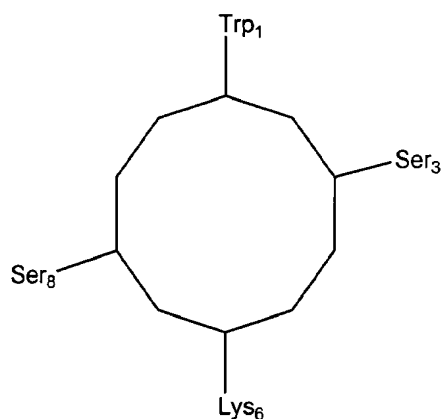
A cyclic peptide is proposed as a structural element that incorporates H-Bond donating and accepting atoms to provide affinity to glucose (and other biomolecules). Careful choice of amino acid residues forming the peptide will allow for adjusting specificity.

Two proof of concept compounds are demonstrated: Cyclic peptides "A" and "B" (Scheme 10).



Cyclic Decapeptide A

WGSGGYGSGG



Cyclic Decapeptide B

WGSGGKGSGG

**Scheme 10.** Proposed cyclic decapeptides for proof of concept experiments.

**Cyclic Decapeptide “A”**

Glucose binding to Peptide A is confirmed by: (1) examination of change in fluorescence polarization of Tryptophan, or (2) changes in energy transfer from Tyrosine to Tryptophan. Note: added serines will boost the binding affinity for Glucose by additional H-bonding sites.

**Cyclic Decapeptide “B”**

Glucose binding to Peptide B is confirmed by one or more of the following: (1) examination of change in fluorescence polarization of Tryptophan, (2) changes in fluorescent quenching of tryptophan by the nitrogen of Lysine, (3) change in fluorescence polarization of tryptophan, or (4) a change in fluorescence polarization or energy transfer of a fluorophore connected to the end of the Lysine side chain. Note: added serines will boost the binding affinity for Glucose by additional H-bonding

Quantitation

SMMR compounds have a number of quantifiable parameters in common. They have high molar absorption coefficients ( $>50,000 \text{ dm}^3\text{mol}^{-1}\text{cm}^{-1}$ ), high fluorescence quantum yield ( $>0.2$ ), and they interact only with components of the cell or biological system that are present in high concentration. This last requirement helps minimize the toxicity of the SMMR. Competitive inhibition of enzymatic processes and interference with cellular processes is minimized if the SMMR does not interact with a significant fraction of the cells metabolic pathways.

**RATIONAL DESIGN FOR NOVEL SMMR COMPOUNDS**

This section discloses the aspects important to intelligent design of SMMRs for glucose detection. There are several individual properties involved during the rational molecular design process and these are disclosed within the following text.

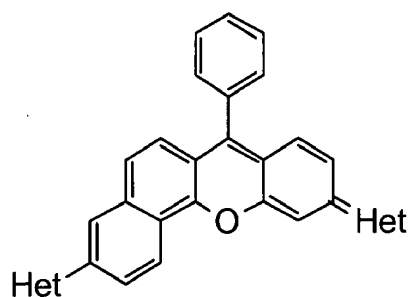
The fluorescence quantum yield ( $\Phi_F$ ) of a molecule is given by equation (12).

$$\phi_F = \frac{k_r}{k_r + k_{nr}} \quad (\text{EQ. 12})$$



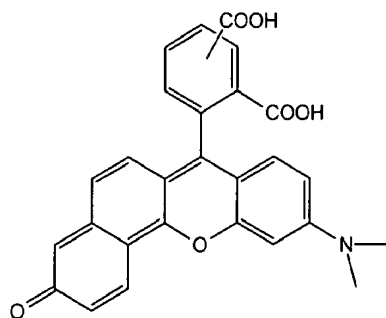
Where  $k_r$  is the radiative rate constant and  $k_{nr}$  is the non-radiative rate constant. In this discussion, the term  $k_{nr}$  includes all mechanisms for decay that does not lead to fluorescence.

The  $\Phi_F$  of BeXan type dyes is typically 0.2 or less. The reasons the value is so low may include the flexibility which increases the number of vibrational modes available to the compound, intramolecular reactions or competing photophysical pathways such as intersystem crossing. All of these processes increase  $k_{nr}$  for deactivation of the excited state. For BeXan it is unlikely that flexibility or intramolecular reactions contribute significantly to  $k_{nr}$ , other molecules of similar size and substitution have  $\Phi_F$  values close to unity. It is more likely that intersystem crossing for the molecule is significant and is the cause for the low  $\Phi_F$ . If the BeXan structure is drawn as shown here then the molecule can be designed with two linked chromophores (Scheme 11, below):



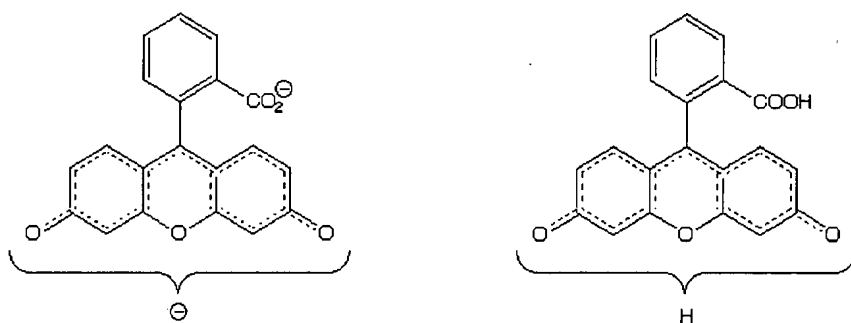
a quinoid type structure involving the heteroatom at position 9 and a substituted naphthalene structure involving the heteroatom at position 3. Het refers to a heteroatom, most commonly oxygen or nitrogen. The photochemistry may be interpreted as a combination of these two chromophores. The validity of this kind of interpretation is dependent on the fraction time the molecule may be considered in this configuration as opposed to a configuration involving a naphthoquinoid and a phenol type structure.

The configuration of the dye is strongly influenced by the nature of the heteroatom substitution. The structure of the dye responsible for the absorption may be estimated by consideration of the photophysical properties of related compounds and calculations on them. For example comparison of fluorescein and BeXan type dyes are shown in Figures 21a and 21b, with structures shown in Schemes 11 and 12, respectively.



**Scheme 12.** Structure of deprotonated BeXan dye shown with no delocalization

5



**Scheme 13.** Deprotonated and protonated forms of fluorescein shown delocalized

10 Table 4 demonstrates the absorption and emission maxima (*i.e.*, wavelength in nanometers) with corresponding energies for fluorescein and BeXan visible spectral absorption bands. The table also demonstrates other spectral properties for a comparison of these two molecular structures.

15 **Table 4.** Absorption and emission position and energy data for fluorescein and BeXan

	Absorption Wavelength (nm)	Emission Wavelength (nm)	Absorption Energy (kcal/mol)	Emission Energy (kcal/mol)
Fluorescein Protonated	455, 483	516, 541	62.8, 59.2	55.4, 52.9
Fluorescein Deprotonated	501	540	57.1	53
BeXan Protonated	518, 549	586, 635	55.2, 52.1	48.8, 45
BeXan Deprotonated	576	640	49.6	44.7

*Difference in energy of protonated absorption bands*

Fluorescein 3.6 kcal/mol

BeXan 3.1 kcal/mol

5

*Stokes shift for deprotonated bands*

Fluorescein 4.1 kcal/mol

BeXan 4.9 kcal/mol

*Stokes shift for protonated bands*

Fluorescein 3.8 kcal/mol

BeXan 3.3 kcal/mol

10

Band positions relative to the high-energy protonated absorption band

15

	Fluorescein (kcal/mol)	
	Absorption	Emission
High energy protonated band	0	7.4
Low energy protonated band	3.6	9.9
Deprotonated band	5.7	9.8

20

	BeXan (kcal/mol)	
	Absorption	Emission
High energy protonated band	0	6.4
Low energy protonated band	3.1	10.2
Deprotonated band	5.6	10.5

25

These two compounds show very similar visible absorption and emission spectra. The absorption spectrum of the BeXan type dye is about 70 nm shifted to the red, probably as a consequence of extended conjugation compared with fluorescein. The emission spectrum of BeXan is shifted by as much as 100 nm compared to fluorescein. Despite this difference in the wavelengths the energy spacing of the visible bands is also similar. There is a slightly larger Stokes shift for BeXan, which may have been expected since the flexibility of the naphthoxanthene structure is likely to be greater than that for the xanthene ring.

30

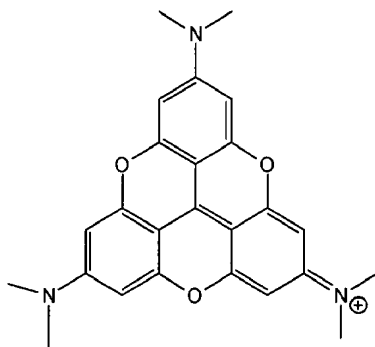
The similarities in these spectra support the idea that in the BeXan ring system there is a high degree of delocalization both in the protonated and the deprotonated form of the dye as is observed for fluorescein. These similarities also support the idea that substitution of other heteroatoms onto the xanthene ring structure should not lead to significant changes to the spectral properties of the molecule. The parameter that cannot be accounted for in this kind of analysis is the quantum yield of fluorescence.

#### Comparison of the Structures of Two Dyes

As an example of the principle, Malachite green is an aryl methine dye that absorbs strongly in the red region of the spectrum ( $150,000 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ ). It is non-fluorescent.

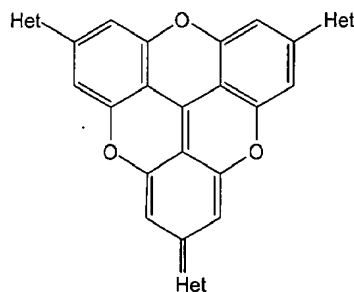
- Conjugation in this molecule probably extends over the three phenyl rings with the positive charge located primarily on the two substituted rings [Figure 22 shows the absorption spectrum (left), and the molecular structures (right)]. Comparison of this structure with that of Rhodamine B shows the two substituted rings tied together with an oxygen bridge [Figure 23 shows the absorption spectrum (left), and the molecular structures (right)]. The molar absorption coefficient is  $109,000 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ . Molecular modeling shows the phenyl ring twisted out of plane with respect to the xanthene ring. A consequence of this is that the molecule has an absorption maximum about 60 nm to the blue, supporting the view that the phenyl ring is not involved in the main xanthene chromophore. The molecule is also significantly more rigid than the aryl methine dye and the quantum yield of fluorescence is close to unity.

Consideration of the structures of these two compounds and their desirable photophysical properties leads to the design of a new chromophore that may well have a very high molar absorption coefficient and a high quantum yield of fluorescence, the structure of which is shown in Scheme 13.



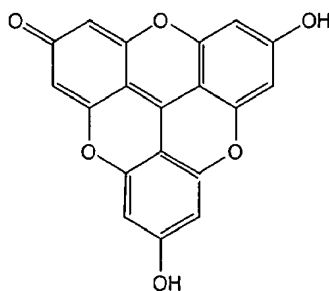
**Scheme 14.** Design structure of a new chromophore proposed to have a very high molar absorption coefficient and a high quantum yield of fluorescence

Molecular modeling of this compound shows it to be highly planar and from earlier arguments it would be expected to absorb at 600 nm or higher and to be highly fluorescent. There is little information concerning this type of structure in the literature but a synthesis has been published of a molecule 2,6,10-Tris(dialkylamino)trioxatriangulenium (2,6,10-Tris(dialkylamino)trioxatriangulenium ions. Synthesis, structure, and properties of exceptionally stable carbenium ions. B.W. Laursen, F.C. Krebs, M.F. Nielsen, K. Bechgaard, J.B. Christensen and N. Harrit. J. Am. Chem. Soc. (1998) 120 12255-12263). The rational behind its synthesis however extends to other molecules with heteroatom substitution including the carbonyl/hydroxyl derivative, the sulfur analog and mixed heteroatom analogs such as that proposed in Scheme 14.



**Scheme 15.** A rational design for heteroatom substitution for the molecule proposed in Scheme 14, including the carbonyl/hydroxyl derivative, the sulfur analog and mixed heteroatom analogs.

To the best of our knowledge there is no previous reference to the oxygen or sulfur analog as proposed in Scheme 15.



*Scheme 16.* An oxygen analog of the molecule as proposed in Scheme 15. Note that a sulfur analog would also be obvious to one skilled in the art from this description.

*Enhancement of cellular retention and quantum yield*

5           The problems that exist for the some dyes that make them unsuitable as SMMRs are low quantum yield of fluorescence, poor retention inside cells, and toxicity. These problems may be addressed to a certain extent by binding the dye to a controlled size metal particle. The fact that the quantum yield for fluorescence is determined by the balance between the radiative and non-radiative rate constants has already been discussed. For dyes that have low quantum  
10   yield of fluorescence the non-radiative rate constant dominates the radiative rate constant. In the presence of a strong electric field, the radiative rate constant may be increased to such an extent that it dominates in determining the quantum yield.

          This phenomenon is well known near the surface of metals. If a fluorophore with a low quantum yield is placed within a certain distance from a metal surface then a dramatic  
15   shortening of the fluorescence lifetime and an enhancement of the fluorescence quantum yield may be observed. If very small (nm) beads are used to bind the dye, or the dye resides on the surface of the metal the dye may stop fluorescing completely. Selecting the optimum bead size and dye spacing from the surface of the metal has some important consequences:

          If the bead is too small or if the dye is so close to the metal that it resides on the surface,  
20   the excited state of the dye is completely quenched. If the particle size is too large, the particle bound dye will not be taken up by the cell.

          The optimum dye-to- particle spacing and particle size will lead to a SMMR system that has a quantum yield close to unity. Because the radiative rate constant is so high the fluorescent lifetime will be short and as a result there will be less time for excited state  
25   chemistry to take place, reducing phototoxicity. Since the dye is bound to a relatively large bead it cannot migrate away from the active site and overall toxicity is reduced. With a short excited state lifetime the photostability of the molecule will be improved for the same reason that the phototoxicity is reduced. The synthesis of these complexes is relatively simple. If the bead is made from gold then the dye will bind with a free thiol group. These molecules may be  
30   prepared starting with the chloromethyl BeXan dye. It is reacted at a 1:1 ratio with a dithioalkane. The free thiol terminus binds to the gold surface.

Effective concentrations of SMMRs to be applied in compositions and methods of the invention are in the range of at least 1 to 500  $\mu\text{g/ml}$ , *e.g.* 5 to 150  $\mu\text{g/ml}$  or 10 to 100  $\mu\text{g/ml}$ . The concentration of SMMRs used is preferably from 10 to 500  $\mu\text{M}$ , more preferably from 100 to 300  $\mu\text{M}$ , and most preferably from 150 to 250  $\mu\text{M}$ .

5 *Techniques for Placement of SMMRs into the Epidermis*

For any of the embodiments described herein, a series of techniques exist that allow the placement of specialized fluorescent or absorptive molecules (SMMRs) into the epidermis, epithelial cells, or peripheral cells (for organs or muscle tissue during invasive surgery). Penetration of the sensor composition can be accomplished using an active transport technique,  
 10 such as, for example, electroporation, laser poration, sonic poration, ultrasonic poration, iontophoresis, mechanical poration, solvent transport, direct application by painting, tattooing methods involving application by needle, an equivalent electrical tattooing technique; or most preferably by using passive transport using special solvent and reporter molecule mixtures. Passive transport may be used to allow small molecules of typically 100 Daltons (Da) to 1000  
 15 Da to enter tissues and cells.

Exemplary methods for passive transport are pressurized delivery and wicking. The method is comprised of a direct measurement of the fluorescence of SMMRs placed within epidermal cells, *i.e.*, keratinocytes. This fluorescence is measured using molecules with specific properties for defining glucose metabolism in epidermis and for inferring the  
 20 magnitude of the change in fluorescence signal to blood glucose concentrations.

Incorporation of a reporter into the tissue without use of an external device is preferred, due to the reduced cost, convenience, and ease of use. Such a passive transdermal delivery solvent system must be accurate and safe. Thus, a more elaborate solvent regime must be applied than that used for the active mechanisms such as tattooing, electroporation, and  
 25 ultrasonic poration. Suitable solvent systems useful for passive transdermal delivery include creams, emulsions, and oils. These solvent systems provide passive transdermal stain delivery into the tissue at a depth of less than 50 microns. The following additives aid the process of tissue penetration for SMMR and create a diffusion rate enhancing solvent system: Soybean Oil, Hazelnut Oil, Jojoba Oil, Sweet Almond Oil, Olive Oil, Calendula Oil, Apricot Kernel Oil,  
 30 Grapeseed Oil, Wheat Germ Oil, refined Light Mineral Oil, Triundecanoin (Akomed C), Undecanoic acid, Caprylic/Capric Glycerides (Akoline MCM), Caprylic/Capric Triglycerides, Propylene glycoldiester of caprylic-/capric acid, Emu oil, all as low viscosity mixtures,

preferably less than 35 cSt at 35 °C. In addition, mixtures of one or more of the above oils in combination with a non-polar dilution solvent can also be used. The solvent system is allowed to passively penetrate the tissue for from about 1 minute, about 5 minutes, about 10 minutes, about 30 minutes to about 2 hours to allow diffusion of the SMMR into the appropriate tissue layer(s).

In addition, penetration of the sensor composition to the desired depth can be accomplished by combining the composition with various molecular size attachments.

After the reporters are injected into, or applied to the surface of the tissue, they are allowed to penetrate in proximity to superficial cells of tissues and organs at a depth from the surface of the cells of from about 10  $\mu\text{m}$  to about 1500 $\mu\text{m}$ . For measurement of specific metabolites, the preferred placement of the reporters should be near the surface of the tissue (*i.e.*, about 10 to about 175  $\mu\text{m}$ ) yet be representative of the overall metabolic state of the tissue in which the reporters are placed. The reporters may also be placed at a greater depth into the tissue. The precise placement of the reporters is controlled by the combination of its molecular properties, including: specific molecular size (*i.e.*, 100 daltons to 100 kilodaltons), polarity, charge, structure, pKa, solubility, the size and type of molecular attachments or anchors, the solvent system used, as well as the specific conditions used for poration (if required). A combination of these factors provides the ability to control the location, diffusion rate, and duration or lifetime of the SMMR within the tissue or organ layers.

The dyes may be introduced into the skin by passive diffusion over a period of 24-48 hours, more preferably over a period of 2-6 hours, and most preferably in 10 seconds to 5 minutes. Contemplated diffusion times include periods less than 48 hrs, 24 hrs, 10 hrs, 6 hrs, 2 hrs, 1 hr, 30 min, 15 min, 10 min, 5 min, 1 min, 30 sec, 10 sec, or 1 sec. With passive absorption, a molecule is placed on the surface of the skin and allowed to penetrate in proximity to the epidermal cells (keratinocytes) directly above the basal layer (stratum basale) at a depth from the surface of skin from 10  $\mu\text{m}$  to 50  $\mu\text{m}$  and up to 175  $\mu\text{m}$  in the pits of the stratum basale extending into the dermis between the dermal papillae. For measurement of glucose, the placement of the SMMR is below the stratum corneum yet above the dermis, more specifically in the stratum spinosum or stratum basale immediately above the upward extensions of the dermal papillae. This SMMR placement is accomplished by varying the combination of the polarity and charge on the SMMR, the size of molecular attachments or anchors, as well as by the polarity and hydrophilicity characteristics of the solvent system. The



specific conditions for poration or passive diffusion for placement of the SMMR in the skin are controllable factors. Using any combination of these factors, it is possible to control the localization of the dye within the skin layers and target cells.

Another embodiment of the reporter application involves the use of a reservoir  
5 containing reporter, which is used to automatically or manually dispense a dose of the reporter mixture topically prior to poration or passive transport. For measurement of metabolites and precursors the reporter is placed in the tissue at a depth of up to 300  $\mu\text{m}$ . A solution of 10-400  $\mu\text{L}$  volume made from 1-50  $\mu\text{M}$  SMMR in a solvent system penetrates into the tissue for some period of time to allow activation following passive diffusion kinetics. Once activated the  
10 change in fluorescence or absorption response of the tissue cells to changes in extracellular and intracellular metabolite or precursor concentrations is monitored directly using an optical reader. Irritant chemicals such as salicylic acid can be used to facilitate the penetration of reporters into skin or peripheral tissue.

In another embodiment, a small disposable film patch composed of polyolefin,  
15 polyester, or polyacrylate and having an SMMR dispersed into a transfer gel applied to the transfer side of the film patch, is used for SMMR application. The patch is applied with the gel side toward the skin and the gel contacts the external surface of the skin. Following the gel application, a poration or passive transfer technique is used to introduce the mixture into the appropriate skin layer(s) (as described above). Another embodiment of the SMMR application  
20 involves the use of a reservoir containing molecular tag or SMMR. This reservoir is used to either automatically or manually dispense a dose of the SMMR mixture topically prior to poration or passive transport. A non-limiting example of a topical dose is a small dot or spot from 100  $\mu\text{m}$  to 5 mm. A smaller area is preferred in most embodiments, but a larger area is also contemplated. For measurement of glucose, the SMMR is placed in the keratinocytes at  
25 30  $\mu\text{m}$  to 50  $\mu\text{m}$  and up to 175  $\mu\text{m}$  so that placement is precisely in the specific layer of the epidermis (*e.g.*, above the dermal papillae and within or above the stratum basale), within a comparatively homeostatic keratinocyte stratum. The molecular tag or SMMR penetrates into the skin for some period of time (depending upon molecular size and solvent mixture used) to allow activation following passive diffusion kinetics (*i.e.*, mass transport). Once activated, the  
30 change in fluorescence response of the skin cells to changes of extracellular and intracellular glucose is monitored directly using an optical reader.

An active mechanism utilizing tissue permeation, electroporation, laser poration, or ultrasonic poration is another procedure for introducing SMMRs into the skin. Pulse lengths for poration technologies are provided below. An example of an ultrasonic poration device includes those manufactured by Sontra Medical Corporation, Cambridge Massachusetts.

5 Sontra and other commercial manufacturers of devices useful for this application have previously described a method for sensing glucose directly in the interstitial fluid surrounding the skin cells by removing fluid or gaining access to removed fluid for analysis. *See e.g.*, JA Tamada, M Lesho and MJ Tierney, "Weekly Feature: Keeping Watch on Glucose - new monitors help fight the long-term complications of diabetes." IEEE Spectrum Online, June 10, 10 2003 at website: <<http://www.spectrum.ieee.org/WEBONLY/publicfeature/apr02/glu.html>> (last visited June 26, 2003). The methods and compositions of the invention do not remove fluid but, rather, place small quantities of solution containing low concentrations of SMMRs into the skin for direct reading of the SMMR fluorescence spectral characteristics as an indication of both epidermal skin and blood glucose levels.

15 For some reporters above 1,000 daltons in size, electroporation may be used to introduce reporter into tissue. Electroporation has been utilized for introducing chemotherapy treatments, for introduction of DNA into living cells and tissues, and broadly recommended for introducing materials into tissues for cosmetic or medical treatment applications. If poration schemes are used, the optimized settings for an electroporation device are achieved by 20 commercially available or by a customizable device having settings that provide conditions as described within this invention. Commercial systems utilizing a square wave voltage pulse have been described within the literature, such as those available from Genetronics Biomedical Corporation, 11199 Sorrento Valley Road, San Diego, CA 92121. Such a small device can be inexpensively made to have one or more constant settings for the optimized conditions 25 disclosed for this invention.

Electroporation uses a short pulse electrical field to alter cell membrane permeability. Micro-pores form in the membrane of skin cells allowing the introduction of various molecular size mixtures into the cells at an appropriate depth of penetration for this specific inventive application. When the electric field is discontinued, the cells return to normal and one or more 30 SMMRs introduced into the cell using the technique remains at the cellular site specifically within the epidermal cell until either the dye is chemically degraded and disposed of within the tissue or is sloughed off in a normal desquamating cycle. The process of sloughing off (or

desquamation) follows a normal ten-day to twenty-day (typically fourteen-day) cycle as the residence time of epidermal keratinocytes moving from the basal layer (stratum basale) to the desquamating layer of the stratum corneum.

When employed, electroporation is optimized for use in this invention by selection of  
 5 voltage range (from about 40 to 90 Volts), gap distance (from about 0 to 2 mm), pulse length (from about 150 to 250 ms), number or pulses (from about 1-10), pulse interval (from about 5 to 60 s), specific electrode design, and desired field strength (from about 40 to 60 V/cm). In addition, the selection of molecular tag molecules, solvent molecules, concentration, and lag times relative to measurement onset is determined as precisely as possible. In certain  
 10 embodiments, specific parameters are determined empirically using specific solvent and SMMR selection. For example, optimization of electroporation involves the following specifications:

1. Output voltage range: 0 to +200 VDC;
- 15 2. Discharge capacitor ( $C_{dis}$ ) values in microfarads are on or about: 200, 500, 700, 1000, 1200, 1500, 1700  $\mu F$ ;
3. Pulse type: exponential decay;
4. Pulse  $R_t C_{dis}$  decay time constant where  $R_t$  (total) =  $5 + R_{skin}$  in parallel with 50 ohms. If  $R_{skin} \gg 50$  ohms then  $R_t = 55$  ohms and  $R_t \times C_{dis} = 11, 27.5, 38.5, 55, 66, 82.5, 93.5$  milliseconds (ms).

20 Electroporation also facilitates the delivery of dyes bound to large molecules that serve as anchors such as polymer beads, large polysaccharides, or colloidal particles. These approaches are contemplated as being within the invention, but are less advantageous in that the particles are often too massive to pass through the stratum corneum without active poration  
 25 or mechanical injection. Once in the skin, they do not readily dissolve or organically reabsorb into the body. Such less desirable approaches would create undesirable particles that would either remain in place indefinitely or accumulate in lymph nodes, in other circulatory cavities and/or in other organ sites.

Reporters of the invention can be made with specific properties such that they are  
 30 retained only within skin cells (keratinocytes) where they report on glycolytic activity and do not harm or affect cellular metabolism. These reporter compounds are sloughed off after a few days, even when permanently integrated into, or attached to, keratinocyte cells. The small quantity of reporter(s) that diffuse away from the epidermis are rapidly degraded within the body and are completely eliminated within a few days. In preferred embodiments,  
 35 reapplication of the reporter(s) is relatively easy to perform. The process of sloughing off (or

desquamating) follows a normal ten-day to twenty-day (typically fourteen-day) cycle as the residence time of epidermal keratinocytes moves from the basal layer (stratum basale) to the desquamating layer of the stratum corneum. Thus, reporters are developed to be applied once every 2 to 3 days, preferably every 3 to 4 days, and more preferably every 5 or more days.

- 5 More advanced solvent systems useful for passive transdermal delivery include, but are not limited to, *e.g.*, creams, emulsions (both oil-in-water and water-in-oil), oils (ointments), gel film patches, a reservoir device, paints, polar solvents and non-polar solvents. Non-polar solvents are preferred, as these are most miscible with the SMMRs of the invention and the stratum corneum lipids cementing the keratinocyte lamellae in place. "Lipid solvent systems"
- 10 have been reported in the literature for use in transdermal drug delivery, and are composed to resemble the chemistry of stratum corneum lipids. Such a mixture may also be used to place the SMMRs into the appropriate point within the epidermis. Such a suggested mixture includes: (w/w): ceramide (50%), cholesterol (28%), palmitic acid (17%) and cholesteryl sulfate (5%). *See, e.g.*, Downing *et al.*: Partition of dodecyl sulfate into stratum corneum lipid
- 15 liposomes. *Arch. Dermatol. Res.* 1993, 285:151-157.

- The objective of each of these solvent systems is to provide passive transdermal SMMR delivery into the skin at a preferred depth of from about 10 to 175  $\mu\text{m}$  (microns), more preferred from about 20 to 100 microns, and most preferred from about 20 to 50 microns. For example, the following solvents as additives to the final SMMR mixtures are added to the skin
- 20 to initiate passive transport of the SMMR to the target cellular site. The materials listed aid the process of skin penetration for SMMRs and create a diffusion rate enhancing solvent system for transdermal delivery: dimethyl sulfoxide, ethanol, isopropanol, chloroform, acetic acid, saturated hydrocarbon solvent (with from 10 to 40 carbons as linear or branched chained molecules), soybean oil, hazelnut oil, jojoba oil, sweet almond oil, olive oil, calendula oil,
- 25 apricot kernel oil, grapeseed oil, wheat germ oil, refined light mineral oil and mineral oil spirits, triundecanoin (akomed C), undecanoic acid, caprylic/capric glycerides (akoline MCM), caprylic/capric triglycerides, propylene glycoldiester of caprylic-/capric acid, and emu oil. All are low viscosity mixtures, preferably less than 35 cSt at 35 °C. In certain embodiments, mixtures of one or more of the above oils are used in combination with a non-polar dilution
- 30 solvent.

Factors that control the depth of penetration of the SMMR and its compartmentalization into the cells and domains of the epidermis include the polarity and partition coefficient of the

SMMR as well as the solvent and the molecular size. The SMMR compound may also be derivatized so that it is readily taken up by the cell and then acted upon by enzymes that chemically alter the SMMR to prevent it from leaking out of the cell. One advantage of this type of approach is that the SMMR is only taken up in its active form by viable cells.

- 5 Predictive schemes for determining appropriate derivatization of SMMR compounds are provided below. Alternative methods of derivatization well known to those skilled in the art are also contemplated as part of the invention.

The physical properties of the solvent system that strongly influence permeability in the skin include the molecular size, the vapor pressure, the water solubility, and the octanol water  
10 coefficient. Smaller molecular size increases the diffusion coefficient. The vapor pressure controls the balance between diffusion into the skin and evaporation from the surface. The water solubility and the octanol water partition coefficient determine the miscibility of the SMMR solution between aqueous interstitial fluid and hydrophobic core of the cell membrane.

For a passive solvent delivery system, the depth of penetration of the SMMR is strongly  
15 dependent on the volume of solvent added. Typically, the volume of SMMR used is from 10  $\mu$ L to less than about 100  $\mu$ L. Preferably, the concentration of SMMR is from 10 to 500  $\mu$ M, more preferably from 100 to 300  $\mu$ M, and most preferably from 150 to 250  $\mu$ M. Target cells are exposed to extracellular concentrations in the range of 1 to 10  $\mu$ M. Dilution of the SMMR concentration arises because of the diffusion properties from the surface of the tissue to the  
20 target cell site.

The proposed volume range added to the skin or other tissue is preferably from 1 to 50  $\mu$ L, more preferably from 5 to 20  $\mu$ L, and most preferably from 5 to 15  $\mu$ L. Alternatively, a gel patch is used containing an SMMR coated surface of approximately 6 mm in diameter consisting of a concentration of SMMR preferably from 10 to 500  $\mu$ M, more preferably from  
25 100 to 300  $\mu$ M, and most preferably from 150 to 250  $\mu$ M.

Solvent systems used for SMMRs may be adjusted depending upon their molecular properties and compatibility with the specific SMMR being delivered. For example, solvent hydrophobicity and polarity are noted along with the solubility properties of the SMMR, which will all have an effect on the movement of the SMMR into the tissue. Each SMMR has a  
30 certain affinity for the solvent and the tissue. The solvent's activity for delivering the SMMR directly to target tissue is a matter for empirical testing. One preferred embodiment of the invention uses an SMMR dissolved in DMSO (dimethyl sulfoxide) and further diluted in a

saturated hydrocarbon solvent (with from 10 to 40 carbons as linear or branched chained molecules), or an alcohol (with from 2 to 4 carbons) at a volume ratio of 5:95 to 20:80, respectively. The optimum volume of DMSO in the delivery solvent is less than 20 percent, as the DMSO is used to facilitate dissolution of the SMMR into the carrier hydrocarbon mixture.

- 5 The mixture is added to the tissue in the concentrations and volumes described above.

A gel patch may be used to apply the SMMR. In one embodiment, a gel contains the SMMR in a volatile hydrocarbon solvent in suspension with a polymer such as PVA (polyvinyl alcohol). When placed against the skin or other living tissue, the heat of the skin causes the SMMR (dissolved in the PVA-hydrocarbon solvent) to diffuse into the skin. The final  
10 diffusion depth is controlled by length of application time. Volumes below 100  $\mu$ L minimize extraneous transdermal delivery and maximize delivery into the epidermis target area. Optimum passive solvent delivery is attained by using a solvent mixture or emulsion that facilitates the movement of SMMR across the stratum corneum into the epidermis, but then dissipates rapidly to limit movement of the SMMR away from the target area. Solvent systems  
15 that have the lowest toxicity include water, saturated hydrocarbon oils, polyethylene glycols and glycerol. Solvents systems that include alcohols and dimethyl sulfoxide are less favored in this application since these solvents are less biologically inert.

The SMMRs are applied directly to the surface of the skin and then passively allowed to penetrate the skin for a period of 1 minute to 5 hours, more preferably less than 4 hours, and  
20 most preferably less than 1 hour. Ideally, a solvent delivery system would be developed to provide SMMR delivery to the target tissue in less than 1 hour, more preferably less than 30 minutes, and most preferably in less than 5 minutes. This time period allows the passive diffusion of the SMMRs into the appropriate epidermal cells.

Once the one or more SMMRs are activated as a result of placement within the skin,  
25 measuring fluorescence monitors the response of the skin cells to glucose. As described herein, the fluorescence mechanism used is either a direct or indirect indication of the glucose concentration in the target cell environment. Fluorescence is typically measured using an optical reader. The optical reader calculates the skin response to glucose, applies first principles mathematical models to the response (as described below and shown in FIG. 7), and  
30 provides a determination of the blood glucose levels (See FIGS. 3, 8-9). The choice of the particular commercially available or custom designed optical reader that is compatible for use

with the methods and compositions of this invention is within the ability of one skilled in the art of the invention.

Instrumentation Required for Reporter Monitoring

The instrumentation required to detect changes in reporter signal may consist of simple  
 5 light emitting diode sources combined with low-cost solid-state detectors. The mechanism of  
 signal extraction relating to a biochemical or physiological process is derived from the  
 elucidation and measurement of key metabolic pathways. The reporters are excited, and the  
 remitted energy detected over the wavelength region of 190 nm to 850 nm (See FIG. 38). The  
 three mechanisms of measurement for metabolites or precursors using the reporters of the  
 10 invention include (1) using reporters to increase the signal-to-noise of native autofluorescence  
 signals indicative of human reductive metabolism [ $\text{FADH}_2$ , NADH, and NAD(P)H], (2) using  
 reporters for selection and enhancement of specific metabolite and precursor signals in tissue  
 that are indicative of metabolic state and allow determination of changes in metabolism [ $\text{Ca}^{2+}$ ,  
 lactate, oxygen], and (3) using reporters to directly measure the presence of intracellular or  
 15 extracellular molecular metabolites [protein-FL, and protein- $^3\text{FAD}^*$ ].

All three mechanisms of signal identification and enhancement allow utilization of  
 low-cost, hand held spectrophotometric equipment (*e.g.*, LED excitation and diode detectors)  
 that is simple in design and does not require advanced or complicated computational  
 algorithms. Such equipment is not harmful to subjects and requires just an additional  
 20 disposable component (other than a calibration strip) to prepare the subject for metabolite  
 monitoring. A measurement device approximately the size of a personal cell phone having  
 quality features, such as those which allow the user to determine whether a specific  
 measurement is valid, or whether a repeat measurement is required, can be used. Such a hand-  
 held, battery powered device is intended to be used either occasionally, or on a continuous,  
 25 real-time monitoring basis for subjects requiring serious health management regimes. A single  
 calibration allows continuous monitoring for up to several hours. A calibration technology that  
 utilizes a calibration strip, which mimics the optical response of the subject and allows freedom  
 from continuous correction using primary analysis devices, can be used. Other calibration  
 technologies contemplated by the invention will be readily discerned by those skilled in the art.

30 As an example, to use the device, the subject or physician prepares the area to be  
 measured using the enhancement technology, which is painless and requires a patch (similar in  
 appearance to a Band-Aid<sup>®</sup> bandage), paint, or spray to be applied to the targeted tissue area.

This treatment conditions the tissue area for from a few minutes up to 30 days, depending upon the SMMR properties selected and the depth at which it has been deposited in the subject tissue. The device is then calibrated using a calibration strip and is ready to make measurements for up to 2 hours or more, without requiring additional calibration. The subject  
5 or physician examines the conditioned area with the sensor and makes a measurement. Typically, the measurement takes less than about 5 seconds, and the sensor provides the appropriate metabolite concentration or reports that a repeat measurement is required.

In another embodiment, if the photophysics of fluorescent dyes are considered, the fluorescence changes associated with the SMMR and the analyte may also be monitored using  
10 fluorescence lifetime technology. One preferred embodiment for such a hand held device capable of measuring lifetime changes is to use a phase and modulation spectrometer, which is a device constructed from a radio frequency modulated light emitting diode and a miniature photomultiplier or photodiode, whose signal is amplified by a phase sensitive amplifier. Such devices have been well characterized in the literature and are commercially available in a  
15 variety of forms. Manufacturers of such devices include: Photon Technology International, Inc., 1009 Lenox Drive, Lawrenceville, NJ 08648; PicoQuant GmbH, Rudower Chaussee 29 (IGZ) 12489 Berlin, Germany; Tecan Systems Inc., 2450 Zanker Road, San Jose, CA 95131; Thermo Oriel, 150 Long Beach Blvd., Stratford, CT 06615. These devices measure both the degree of modulation of the fluorophore and the phase shift of the emission relative to  
20 the excitation light, and these two parameters are then related to the lifetime of the dye. Determination of these parameters at a number of frequencies increases the accuracy of the device.

#### EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than  
25 routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of the invention. Various substitutions, alterations, and modifications may be made to the invention without departing from the spirit and scope of the invention. Other aspects, advantages, and modifications are within the scope  
30 of the invention. The contents of all references, issued patents, and published patent applications cited throughout this application are hereby incorporated by reference. The



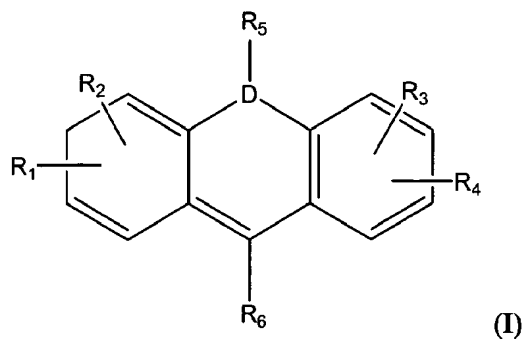
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appropriate components, processes, and methods of those patents, applications and other documents may be selected for the invention and embodiments thereof.

## CLAIMS

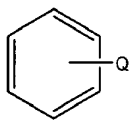
*What is claimed is:*

1. A small molecule metabolite reporter compound of the following formula:



5 wherein

- D is a heteroatom;
- R<sub>1</sub> and R<sub>2</sub> are different and are selected from the group consisting of H, OH, NH<sub>2</sub>, NO<sub>2</sub>, OCH<sub>3</sub>, N(CH<sub>3</sub>)<sub>2</sub>, A, or R<sub>1</sub> and R<sub>2</sub>, taken together with the ring to which they are attached, form R<sub>7</sub>;
- 10 • R<sub>3</sub> and R<sub>4</sub> are different and are selected from the group consisting of H,  $\text{C}(=\text{O})\text{OH}$ , B(OH)<sub>2</sub>, M, or R<sub>3</sub> and R<sub>4</sub>, taken together with the ring to which they are attached, form R<sub>8</sub>;
- R<sub>5</sub> and R<sub>6</sub> are different and are selected from the group consisting of H or

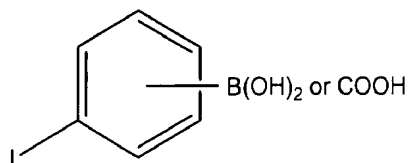
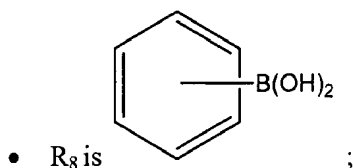


, wherein Q is H, COOH, B(OH)<sub>2</sub>, or M;

15

- A is OH, NH<sub>3</sub>,  $\text{C}(=\text{O})\text{NH}_2$  or  $\text{N}^+(\text{CH}_3)_2$  ;

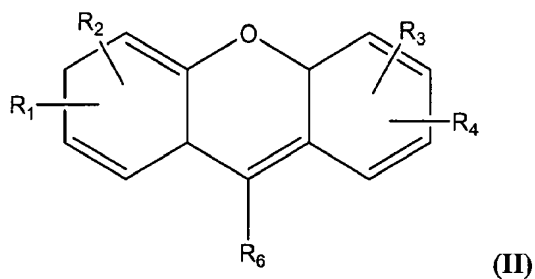
- R<sub>7</sub> is ;



- M is ;
- L, when present, is an amino-containing linking moiety;
- $\text{R}_1$  and  $\text{R}_2$ , and  $\text{R}_3$  and  $\text{R}_4$ , are adjacent to each other on the rings on which they reside; and
- at least one boronic acid moiety is present; and salts thereof.

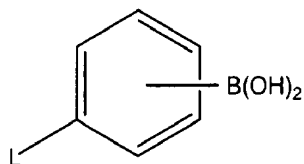
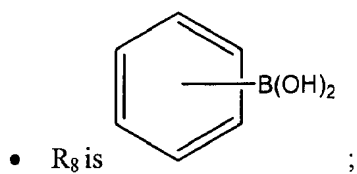
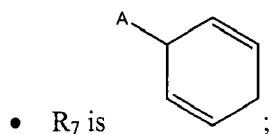
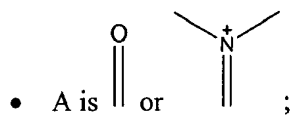
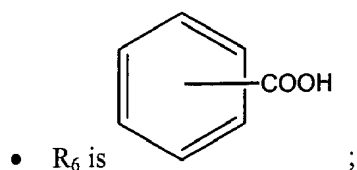
2. The reporter compound of claim 1, wherein D is N or O.

3. A small molecule metabolite reporter compound of the following formula:



wherein

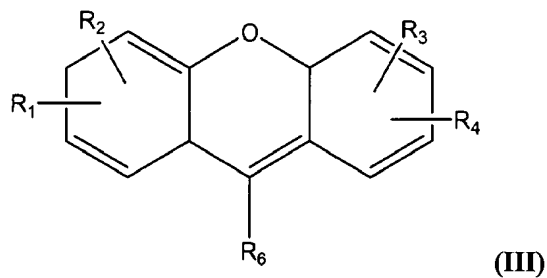
- $\text{R}_1$  and  $\text{R}_2$  are different and may be A, or,  $\text{R}_1$  and  $\text{R}_2$ , taken together with the ring to which they are attached, form  $\text{R}_7$ ;
- $\text{R}_3$  and  $\text{R}_4$  are different and are selected from the group consisting of  $\text{H}$ ,  $\text{C(=O)}$ ,  $\text{OH}$ ,  $\text{B(OH)}_2$ , M, or  $\text{R}_3$  and  $\text{R}_4$ , taken together with the ring to which they are attached, form  $\text{R}_8$ ;



5

- M is ;
- L, when present, is an amino-containing linking moiety; and
- $R_1$  and  $R_2$ , and  $R_3$  and  $R_4$ , are adjacent to each other on the rings on which they reside; and salts thereof.

4. A small molecule metabolite reporter compound of the following formula:




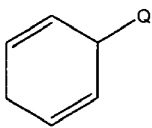
10

wherein


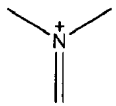
- $R_1$  and  $R_2$  are different and are selected from the group consisting of H, OH,  $\text{NH}_2$ ,  $\text{NO}_2$ ,  $\text{OCH}_3$ ,  $\text{N}(\text{CH}_3)_2$ , A, or,  $R_1$  and  $R_2$ , taken together with the ring to which they are attached, form  $R_7$ ;

5

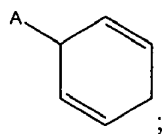
- $R_3$  and  $R_4$  are different and are selected from the group consisting of H, , OH, M, or  $R_3$  and  $R_4$ , taken together with the ring to which they are attached, form  $R_8$ ;
- $R_5$  and  $R_6$  are different and are selected from the group consisting of H or

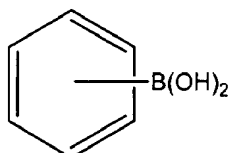


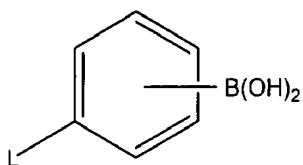
, wherein Q is H or M;

- A is OH,  $\text{NH}_3$ ,  or  ;

10

- $R_7$  is  ;

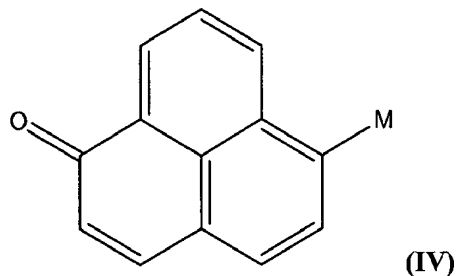
- $R_8$  is  ;

- M is  ;

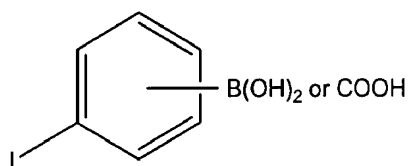
- L, when present, is an amino-containing linking moiety; and
- $R_1$  and  $R_2$ , and  $R_3$  and  $R_4$ , are adjacent to each other on the rings on which they reside; and salts thereof.

15

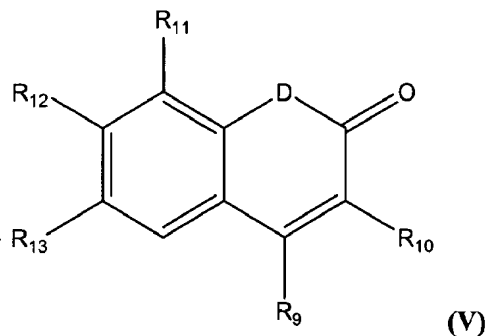
5. A small molecule metabolite reporter compound of the following formula:



wherein

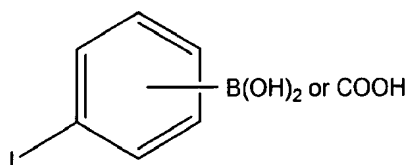


- M is ; and
  - L, when present, is an amino-containing linking moiety; and salts thereof.
6. A small molecule metabolite reporter compound of the following formula:



wherein

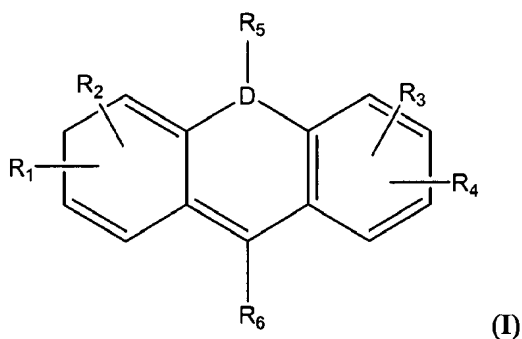
- D is a heteroatom (*e.g.*, O or N);
- R<sub>9</sub> is H, OH, CH<sub>3</sub>, CF<sub>3</sub>, M, or an amino or substituted amino group;
- R<sub>10</sub> is H, CH<sub>3</sub>, or M;
- R<sub>11</sub>, R<sub>12</sub>, and R<sub>13</sub> are individually H, OH, alkoxy, M, or an amino or substituted amino group;
- R<sub>14</sub>, when present, is H or CH<sub>3</sub>;



- $M$  is ; and
- at least one boronic acid moiety is present; and salts thereof.

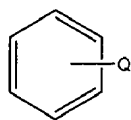
7. A topical sensor composition comprising a compound of any one of claims 1 through 6, and a carrier or binder:

- 5 8. A method of measuring a compound or metabolite thereof, comprising contacting a reporter compound of the following formula:


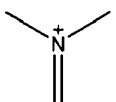


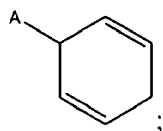
wherein

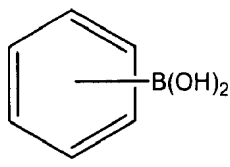
- $D$  is a heteroatom;
- $R_1$  and  $R_2$  are different and are selected from the group consisting of  $H$ ,  $OH$ ,  $NH_2$ ,  $NO_2$ ,  $OCH_3$ ,  $N(CH_3)_2$ ,  $A$ , or,  $R_1$  and  $R_2$ , taken together with the ring to which they are attached, form  $R_7$ ;
- $R_3$  and  $R_4$  are different and are selected from the group consisting of  $H$ ,  $\overset{O}{\parallel}, OH$ ,  $B(OH)_2$ ,  $M$ , or  $R_3$  and  $R_4$ , taken together with the ring to which they are attached, form  $R_8$ ;
- $R_5$  and  $R_6$  are different and are selected from the group consisting of  $H$  or

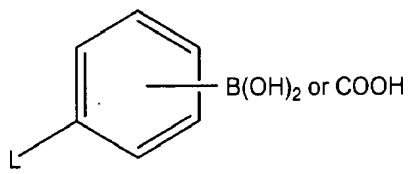


, wherein  $Q$  is  $H$ ,  $COOH$ ,  $B(OH)_2$ , or  $M$ ;

- A is OH, NH<sub>3</sub>,  or  ;

- R<sub>7</sub> is  ;

- R<sub>8</sub> is  ;

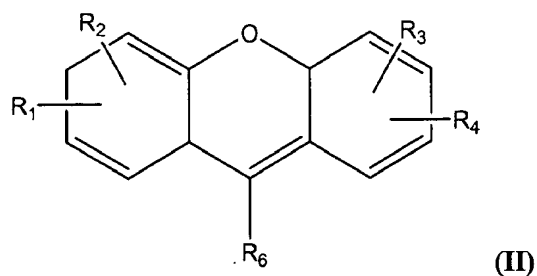
- M is  ;

5

- L, when present, is an amino-containing linking moiety;
- R<sub>1</sub> and R<sub>2</sub>, and R<sub>3</sub> and R<sub>4</sub>, are adjacent to each other on the rings on which they reside; and
- at least one boronic acid moiety is present; and salts thereof, which reporter compound is sensitive to the presence of said compound or metabolite thereof, with an area of the body where said metabolites may be found, and detecting a photometric change in said reporter compound indicative of said metabolite.

10

9. A method of measuring a compound or metabolite thereof, comprising contacting a reporter compound of the following formula:



15

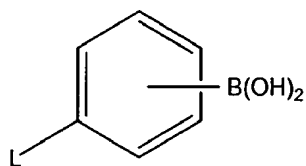
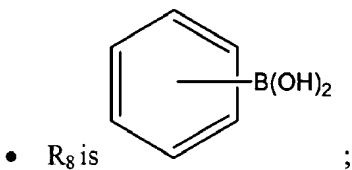
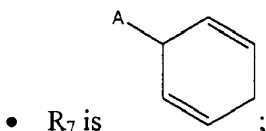
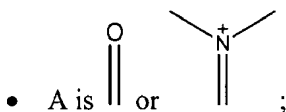
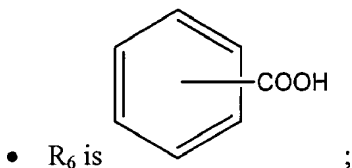
wherein



- $R_1$  and  $R_2$  are different and may be A, or,  $R_1$  and  $R_2$ , taken together with the ring to which they are attached, form  $R_7$ ;

- $R_3$  and  $R_4$  are different and are selected from the group consisting of H,  $\overset{\text{O}}{\parallel}$ , OH,  $\text{B(OH)}_2$ , M, or  $R_3$  and  $R_4$ , taken together with the ring to which they are attached, form  $R_8$ ;

5

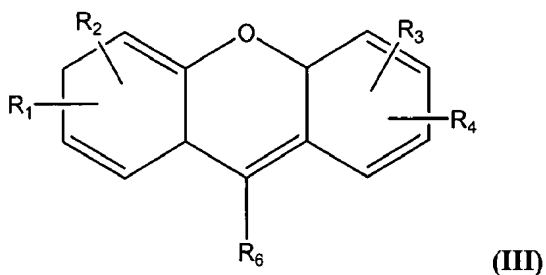


10

- M is ;
- L, when present, is an amino-containing linking moiety; and
- $R_1$  and  $R_2$ , and  $R_3$  and  $R_4$ , are adjacent to each other on the rings on which they reside; and salts thereof; which reporter compound is sensitive to the presence of said compound or metabolite thereof, with an area of the body where said metabolites may be found, and detecting a photometric change in said reporter compound indicative of said metabolite.

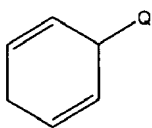
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10. A method of measuring a compound or metabolite thereof, comprising contacting a reporter compound of the following formula:



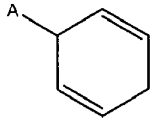
wherein

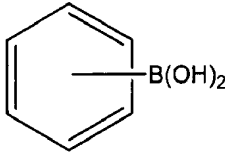
- 5
- $R_1$  and  $R_2$  are different and are selected from the group consisting of H, OH,  $\text{NH}_2$ ,  $\text{NO}_2$ ,  $\text{OCH}_3$ ,  $\text{N}(\text{CH}_3)_2$ , A, or  $R_1$  and  $R_2$ , taken together with the ring to which they are attached, form  $R_7$ ;
  - $R_3$  and  $R_4$  are different and are selected from the group consisting of H,  $\text{C}(=\text{O})$ , OH, M, or  $R_3$  and  $R_4$ , taken together with the ring to which they are attached, form  $R_8$ ;
  - $R_5$  and  $R_6$  are different and are selected from the group consisting of H or
- 10



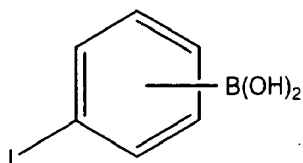
, wherein Q is H or M;

- A is OH,  $\text{NH}_3$ ,  $\text{C}(=\text{O})$  or  $\text{N}^+(\text{CH}_3)_2$  ;

- $R_7$  is  ;

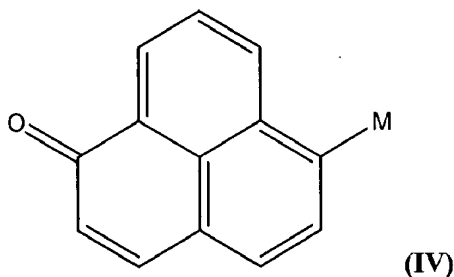
- $R_8$  is  ;

15

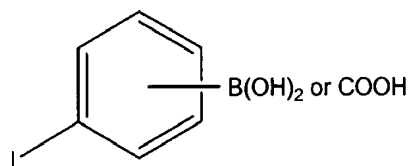


- M is ;
- L, when present, is an amino-containing linking moiety; and
- R<sub>1</sub> and R<sub>2</sub>, and R<sub>3</sub> and R<sub>4</sub>, are adjacent to each other on the rings on which they reside; and salts thereof; which reporter compound is sensitive to the presence of said compound or metabolite thereof, with an area of the body where said metabolites may be found, and detecting a photometric change in said reporter compound indicative of said metabolite.

11. A method of measuring a compound or metabolite thereof, comprising contacting a reporter compound of the following formula:

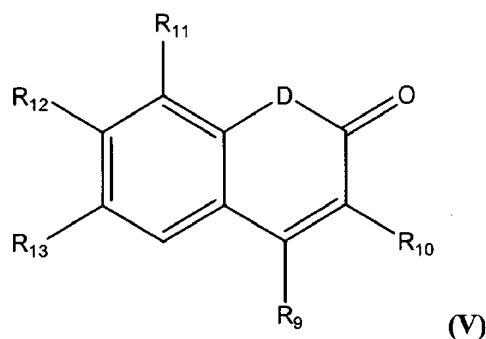


wherein



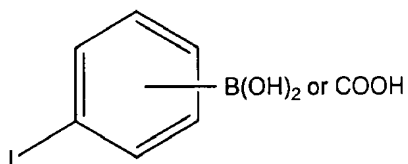
- M is ; and
- L, when present, is an amino-containing linking moiety; and salts thereof; which reporter compound is sensitive to the presence of said compound or metabolite thereof, with an area of the body where said metabolites may be found, and detecting a photometric change in said reporter compound indicative of said metabolite.

12. A method of measuring a compound or metabolite thereof, comprising contacting a reporter compound of the following formula:



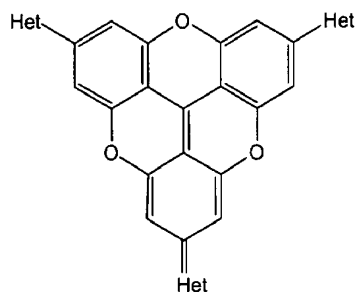
wherein

- 5
- D is a heteroatom (*e.g.*, O or N);
  - R<sub>9</sub> is H, OH, CH<sub>3</sub>, CF<sub>3</sub>, M, or an amino or substituted amino group;
  - R<sub>10</sub> is H, CH<sub>3</sub>, or M;
  - R<sub>11</sub>, R<sub>12</sub>, and R<sub>13</sub> are individually H, OH, alkoxy, M, or an amino or substituted amino group;
- 10
- R<sub>14</sub>, when present, is H or CH<sub>3</sub>;



- 15
- M is ; and
  - at least one boronic acid moiety is present; and salts thereof, which reporter compound is sensitive to the presence of said compound or metabolite thereof, with an area of the body where said metabolites may be found, and detecting a photometric change in said reporter compound indicative of said metabolite.
13. The method of any one of claims 8 through 12, wherein said area of the body is skin.
14. The method of any one of claims 8 through 12, wherein said area of the body is the layer of the skin known as *stratum corneum*.

15. The method of any one of claims 8 through 12, wherein said area of the body is the layer of the skin known as *epidermis*.
16. The method of any one of claims 8 through 12, wherein said area of the body is the layer of the skin known as *dermis*.
- 5 17. The method of any one of claims 8 through 12, wherein said compound is glucose.
18. A chromophore of the following formula:



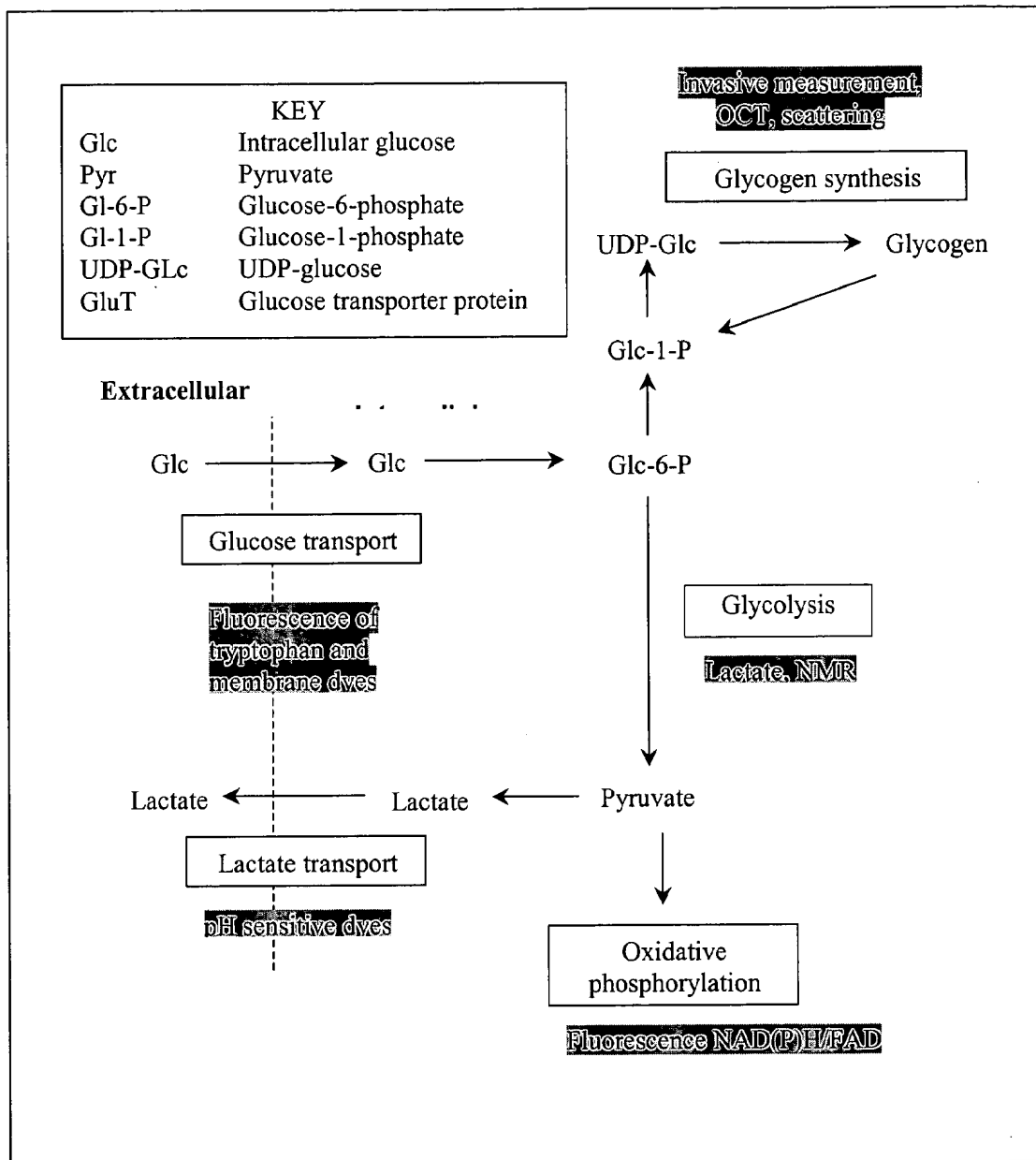
wherein Het represents a heteroatomic group, which groups may be identical or different.

- 10 19. The chromophore of claim 18, wherein heteroatomic group comprises N, O, or S.

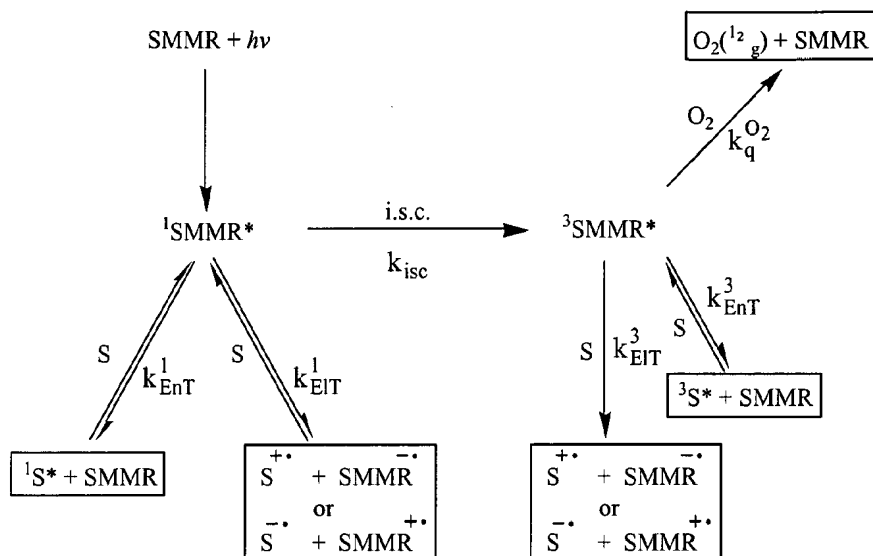
**SMMR (SMALL MOLECULE METABOLITE REPORTERS) FOR USE AS *IN VIVO*  
GLUCOSE BIOSENSORS**

**ABSTRACT OF THE DISCLOSURE**

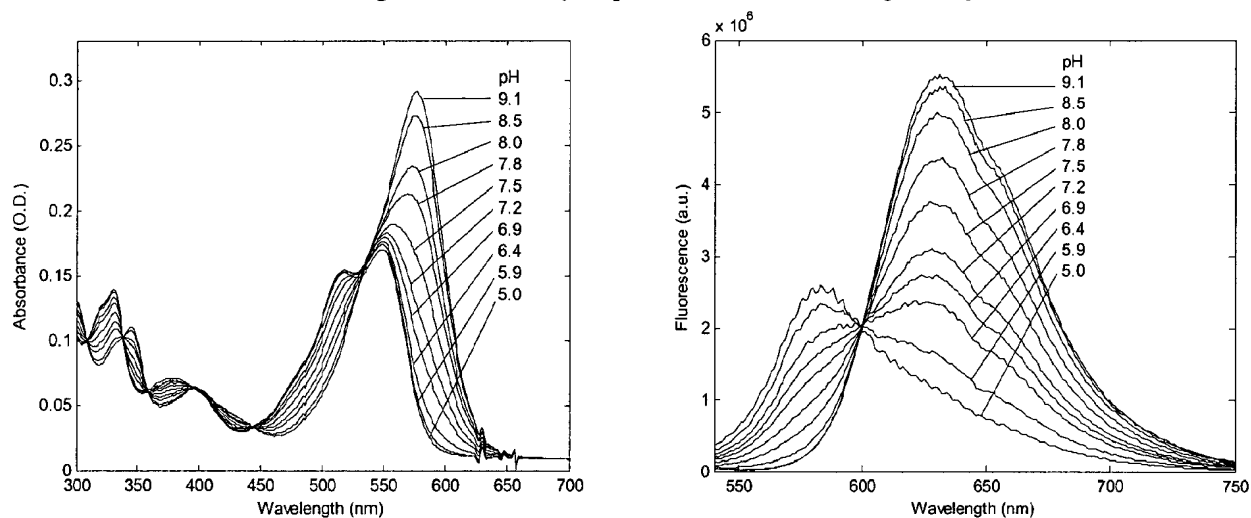
Small Molecule Metabolite Reporters (SMMRs) for use as *in vivo* glucose biosensors,  
5 sensor compositions, and methods of use, are described. The SMMRs include boronic acid-  
containing xanthene, coumarin, carbostyryl and phenalene-based small molecules which are  
used for monitoring glucose *in vivo*, advantageously on the skin.



*Figure 1.* Glucose pathway of living cells

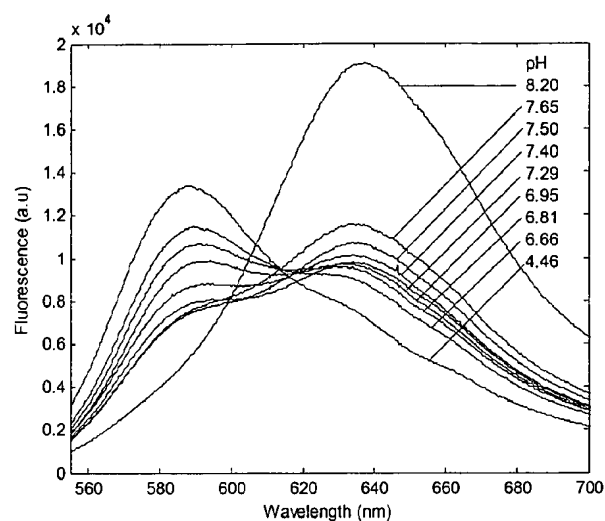


**Figure 2.** Summary of photochemical reaction pathways



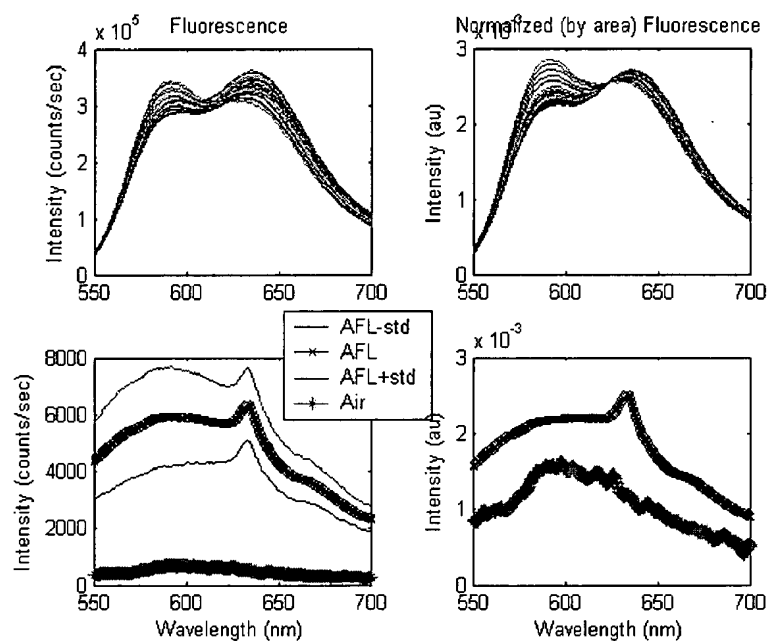
**5 Figure 3.** Absorption and emission spectra of 5  $\mu\text{M}$  of an example ratiometric pH reporting dye in PBS. Fluorescence spectra were measured using 532 nm to excite dye.



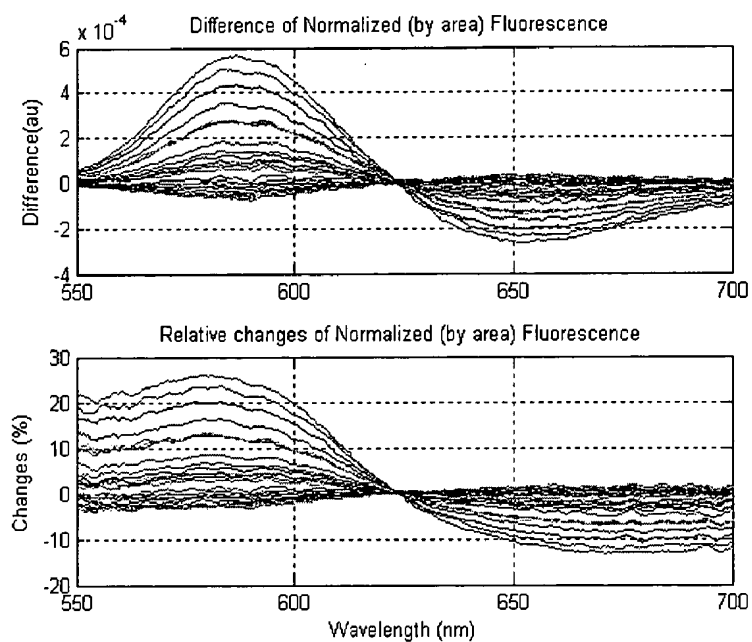


**Figure 4.** Fluorescence emission spectra of an example ratiometric pH reporting dye loaded into the A-431 cells. Nigericin is present and allows intracellular pH to equilibrate extracellular pH.

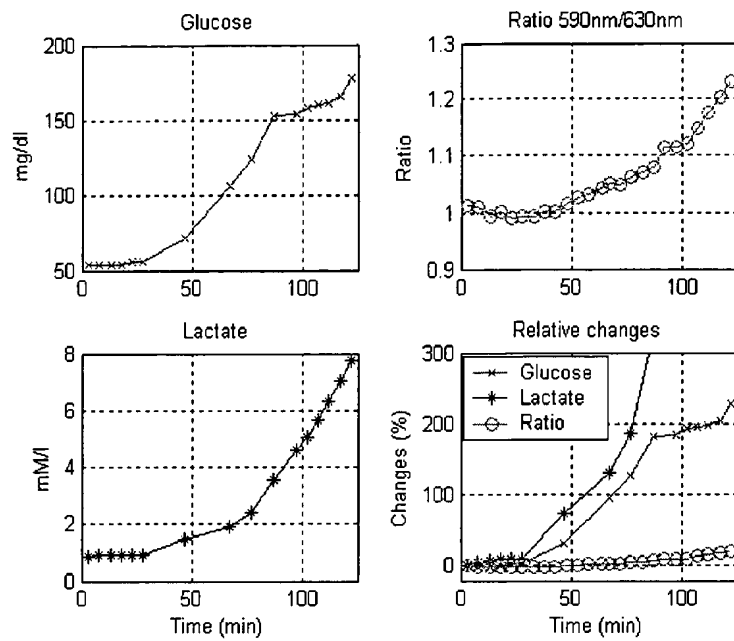
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**Figure 5.** “Absolute” and normalized Fluorescence, auto-Fluorescence and Air spectra.



**Figure 6.** Difference and relative spectra of normalized Fluorescence spectra.



**Figure 7.** Glucose, Lactate and SMMR measured ratio kinetics.

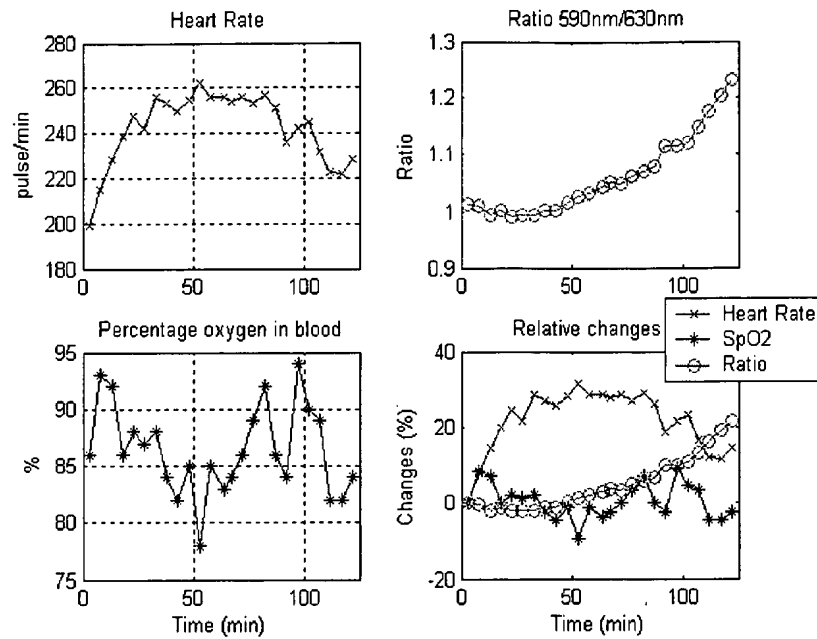


Figure 8. Kinetics of physiological parameters and ratio.

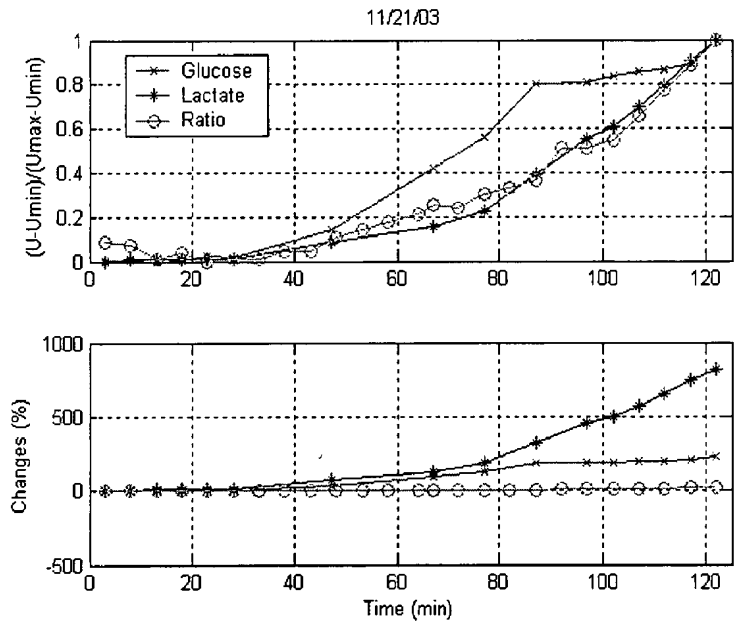


Figure 9. Normalized and relative changes of ratio, glucose, and lactate in time

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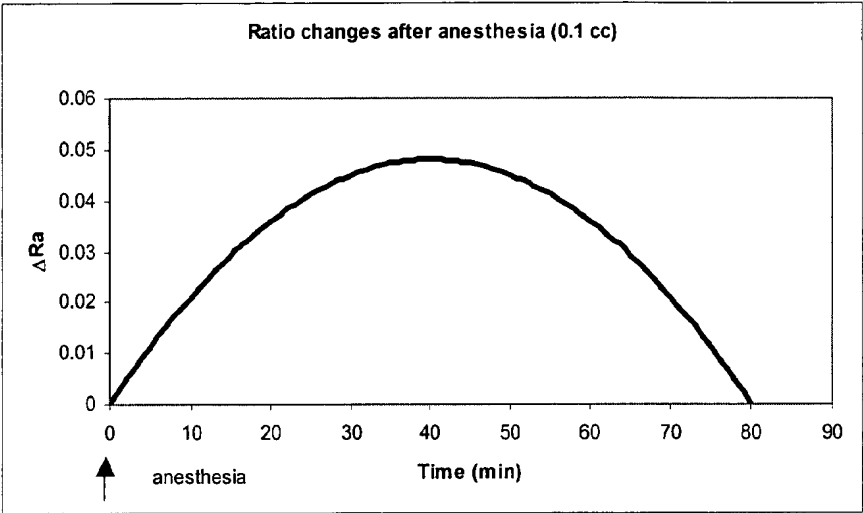


Figure 10. Fluorescence ratio changes after anesthesia (0.1 cc of ketamine).

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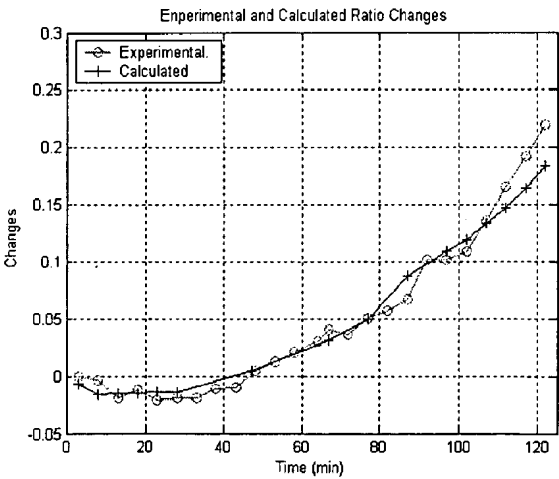
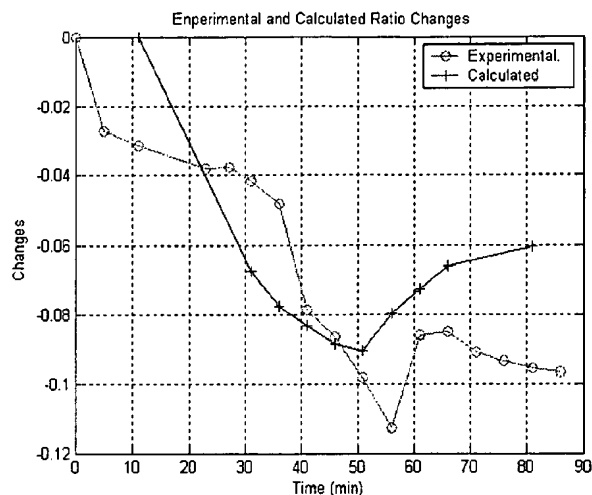


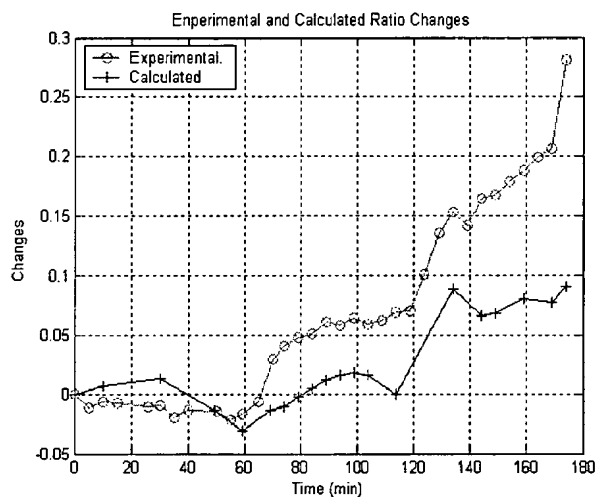
Figure 11a. Data from clamp study #1, comparison between experimental (YSI values) and calculated values of  $\Delta R$  (from the equation  $\Delta R = \alpha \Delta G + \beta \Delta L$  using the coefficients  $\alpha$  and  $\beta$  from above

35



**Figure 11b.** Data from clamp study #2, comparison between experimental (YSI values) and calculated values of  $\Delta R$  (from the equation  $\Delta R = \alpha \Delta G + \beta \Delta L + \Delta R_a(t-t_a)$ ) A using the coefficients  $\alpha$ ,  $\beta$  and  $\Delta R_a(t-t_a)$  from above

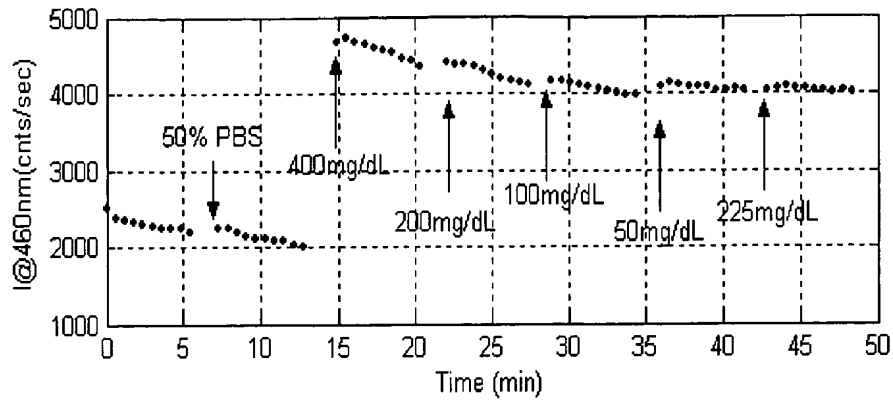
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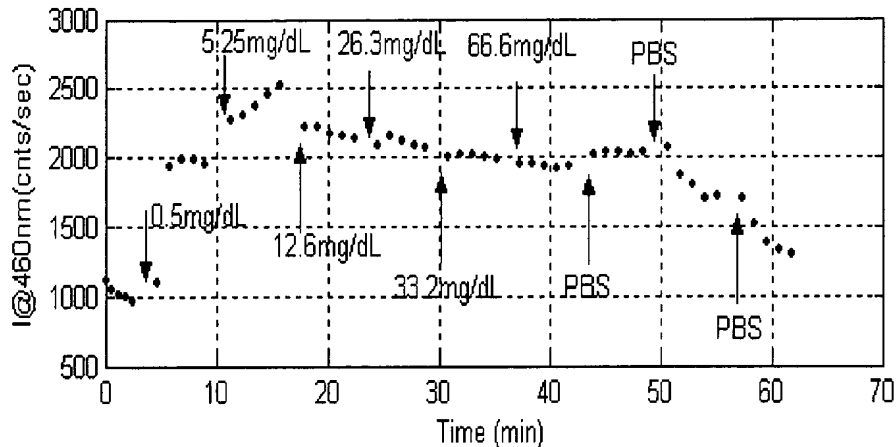
**Figure 11c.** Data from clamp study #3, comparison between experimental (YSI values) and calculated values of  $\Delta R$  (from the equation  $\Delta R = \alpha \Delta G + \beta \Delta L + \Delta R_a(t-t_a)$ ) A using the coefficients  $\alpha$ ,  $\beta$  and  $\Delta R_a(t-t_a)$  from above.

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26069-530 PRO

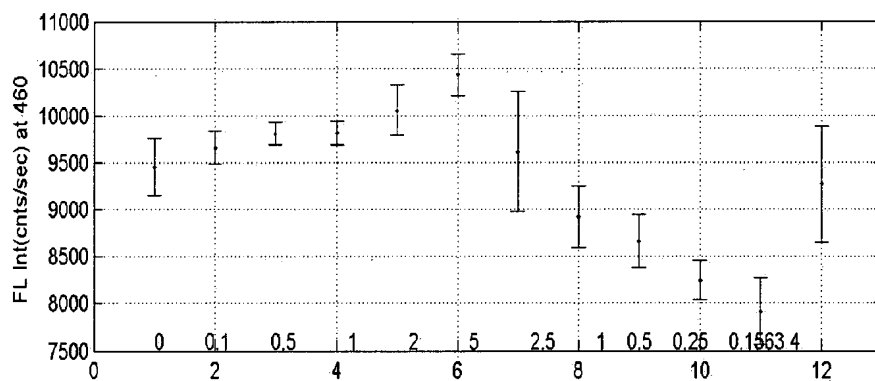


**Figure 12.** NADH peak fluorescence signals versus time as glucose concentration is changed. Glucose concentrations are annotated in the figure. The first annotation with 50% PBS is a control measurement.



**Figure 13.** Glucose concentration is varied from 0 to 66 mg/dL and the NADH peak signals exhibit corresponding changes in intensity.

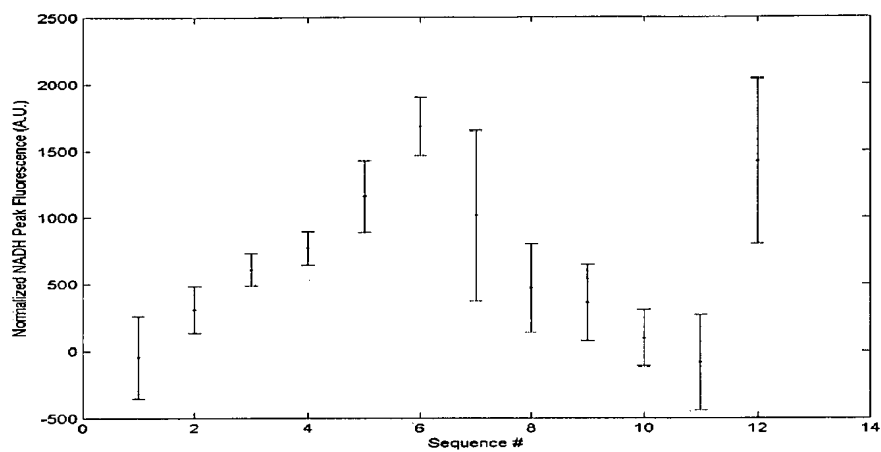
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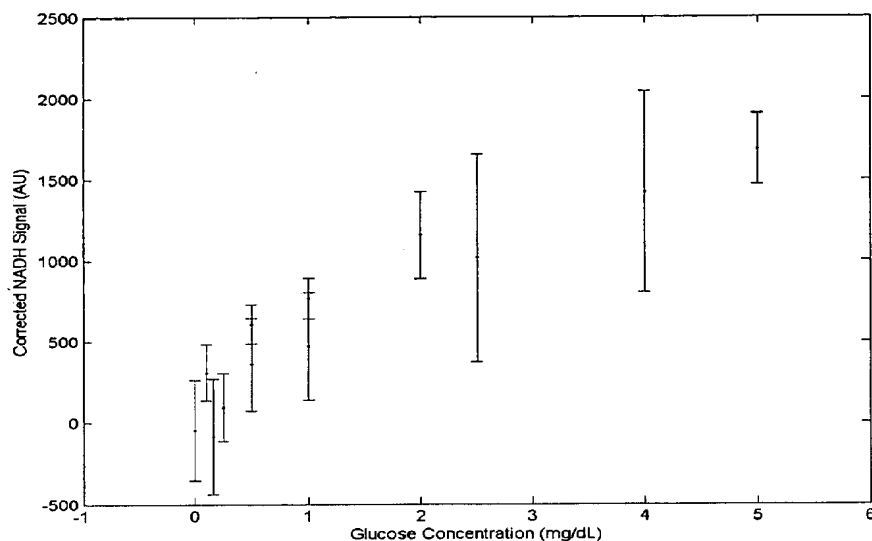
**Figure 14.** NADH measurements versus sequence number. The glucose values (in mg/dL) for each data point are (from left to right): 0, 0.1, 0.5, 1.0, 2.0, 5.0, 2.5, 1.0, 0.5, 0.25, 0.16, and 4.0 mg/dL. Each point represents the mean and standard deviation of 5 data points. NOTE: The x-axis label sequence number.

10



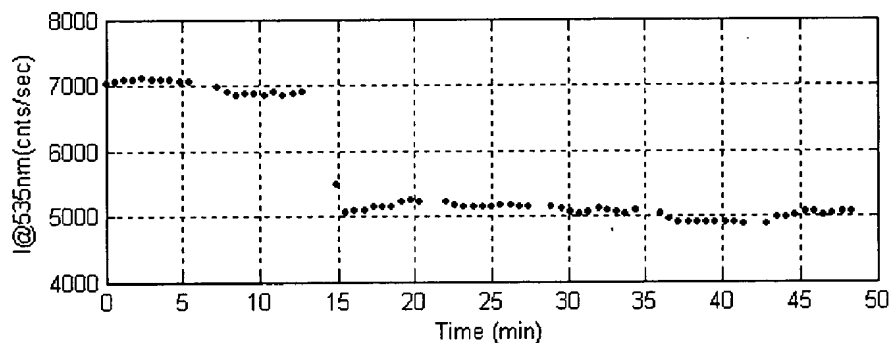


**Figure 15.** Corrected NADH Signal of Figure 14 for each data point (as from left to right), corresponding to a concentration 0, 0.1, 0.5, 1.0, 2.0, 5.0, 2.5, 1.0, 0.5, 0.25, 0.16, and 4.0.



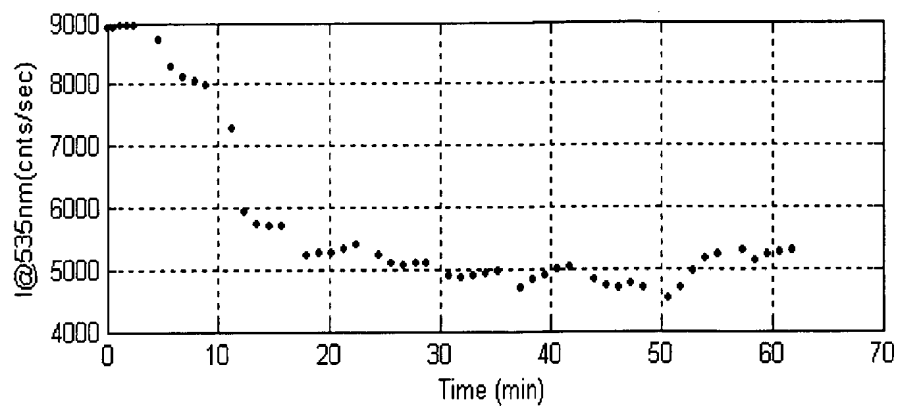
**Figure 16.** Corrected NADH Signal versus Glucose concentration for upward trend and downward trend data together.

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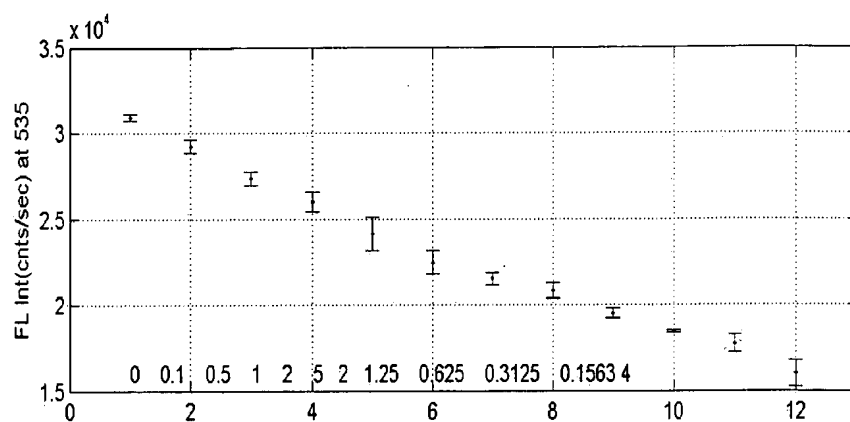


**Figure 17.** A membrane-bound Rh123 peak fluorescence signal change (decrease in signal as an indication of membrane potential change) versus time as glucose concentration is changed. Glucose concentrations were increased at the 15-minute point in the figure. The first annotation with 50% PBS is a control measurement.

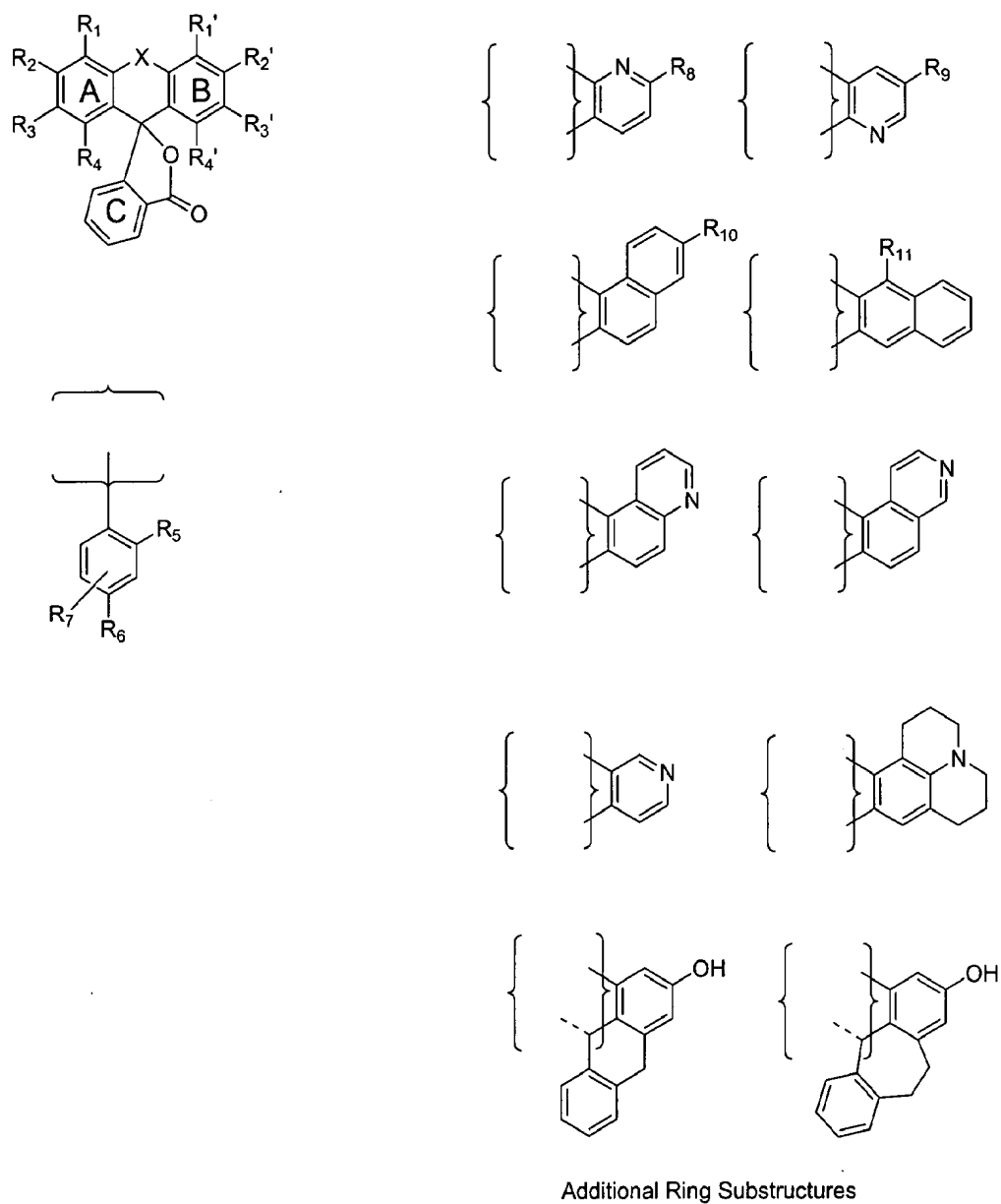
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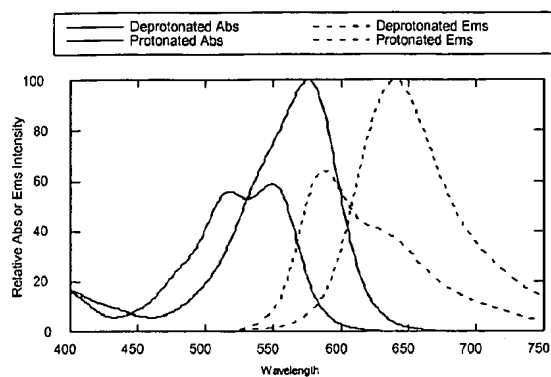
**Figure 18.** Glucose concentration is varied from 0 to 66 mg/dL and the Rh123 (bottom plot) peak signals exhibit corresponding changes in intensity due to membrane potential changes.



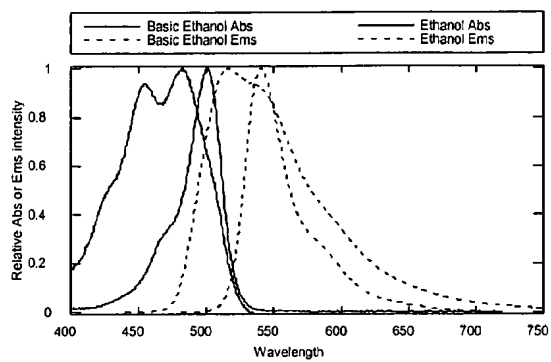
5 **Figure 19.** Rh123 fluorescence quenching (as an indication of membrane potential changes) versus sequence number and glucose concentration. The glucose values (in mg/dL) for each data point is (from left to right): 0, 0.1, 0.5, 1.0, 2.0, 5.0, 2.5, 1.0, 0.5, 0.25, 0.16, and 4.0. Each point represents the mean and standard deviation of 5 data points. The x-axis label is sequence number.



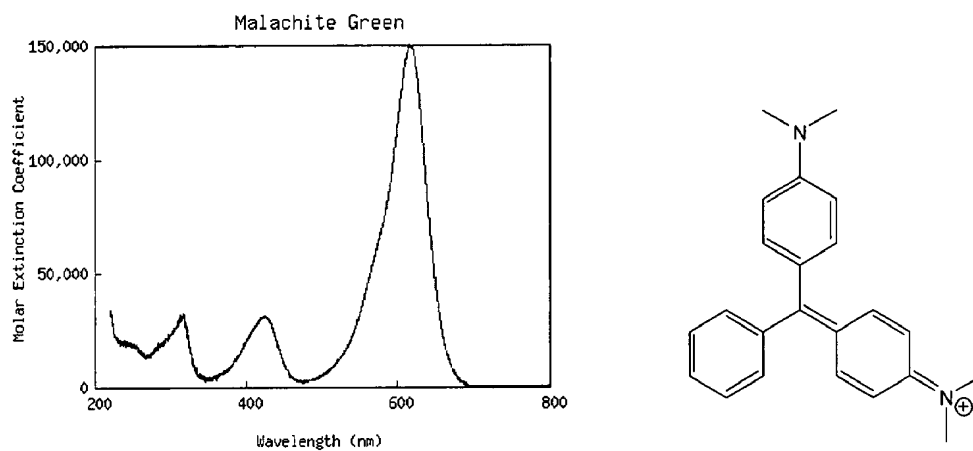
**Figure 20.** Schematic representation of the products of the Xanthene Library in terms of Markush structures and some specific sub structures



**Figure 21a.** Absorption and emission spectra for BeXan type dyes

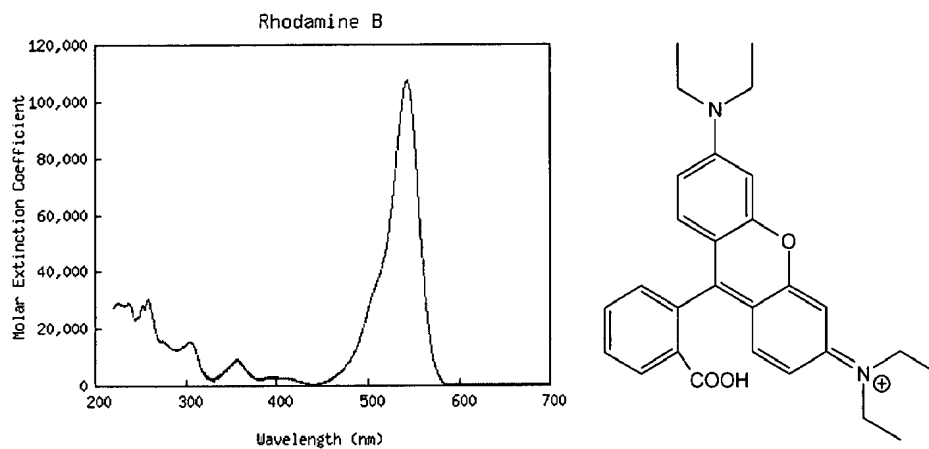


*Figure 21b.* Absorption and emission spectra for fluorescein



**Figure 22.** Malachite green absorption spectrum (left), with molecular structure (right).





**Figure 23.** Rhodamine B absorption spectrum (left), with molecular structure (right).